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Prevention by Cystine or Methionine of Hemorrhage and Necrosis of the Liver in Rats.

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Necrosis, hemorrhage and cirrhosis of the liver in rats were described by György and Goldblatt.[1] They attributed these lesions to a deficiency of a part of the vitamin B_2 complex. By modifying the diet they were later[2] able to increase the incidence of the liver injury. In the report on this work, they stated that the pathogenesis of the necrosis and cirrhosis was related to the lipotropic effect of casein. Blumberg and McCollum[3] reported the production of liver cirrhosis in rats with or without accompanying necrosis and the prevention of the cirrhosis with choline.

Daft, Sebrell and Lillie[4] described the production of liver cirrhosis in rats and its prevention by choline, methionine or casein. Lowry,

1 György, P., and Goldblatt, H., *J. Exp. Med.*, 1939, **70**, 185.

2 György, P., and Goldblatt, H., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 492.

3 Blumberg, H., and McCollum, E. V., *Science*, 1941, **93**, 598.

4 Daft, F. S., Sebrell, W. H., and Lillie, R. D., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 228.

Daft, Sebrell, Ashburn and Lillie[5] reported that treatment by choline or casein of rats with experimentally produced liver cirrhosis resulted in hyperplastic regeneration of liver cells and clinical improvement of the animals. In a paper describing the histology and histogenesis of this liver cirrhosis, Lillie, Ashburn, Sebrell, Daft and Lowry[6] stated that among the noteworthy features is the absence of hemorrhage and, in the earlier stages, of evident necrotic liver cells.

The composition of the diets used in these 3 laboratories is given in Table I. It will be noted that our cirrhosis-producing diet No. 545 contains a much lower percentage both of casein and of fat than the diets of György and Goldblatt[1, 2] and those of Blumberg and McCollum[3] and that our diet contains cornstarch while the others contain sucrose. Diet No. 545 also contains free cystine while the others do not. In view of the classic observations of Osborne and Mendel,[7] it is apparent that the 10% casein diets are deficient in the sulfur-containing amino acids. Recent work of Mulford and Griffith[8] indicates that even the 18% casein diet may be somewhat deficient in these amino acids. It is further apparent that all of these diets except the earlier one of György and Goldblatt[1] are low in protein and that all are low in choline. Diet No. 545 is probably not as low in choline as the others because of the appreciable choline content of cornstarch.[9]

In view of the differences in the diets and the concomitant dif-

TABLE I.
Composition of Diets.

	György and Goldblatt		Blumberg and McCollum[3]		Daft, Sebrell and Lillie[4]
	(1939)[1] Diet	(1941)[2] Diet	Diet (A)	Diet (B)	Diet No. 545
Casein	18	10	10	10	4.0
Cystine					0.5
Sucrose	68	64	29	14	
Cornstarch					86.5
Butter fat	8				
Lard		20	51	66	
Cod liver oil	2	2	2	2	2.0
Corn oil			2	2	
Wesson oil					3.0
Salt mixture	4	4	6	6	4.0

[5] Lowry, J. V., Daft, F. S., Sebrell, W. H., Ashburn, L. L., and Lillie, R. D., *Pub. Health Rep.*, 1941, **56**, 2216.

[6] Lillie, R. D., Ashburn, L. L., Sebrell, W. H., Daft, F. S., and Lowry, J. V., *Pub. Health Rep.*, 1942, **57**, 502.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, **20**, 351.

[8] Mulford, D. J., and Griffith, W. H., *J. Nutrition*, 1942, **23**, 91.

[9] Fletcher, J. P., Best, C. H., and Solandt, O. M., *Biochem. J.*, 1935, **29**, 2278.

ferences in the results obtained in the three laboratories, it seemed advisable to study the effect of variations in the dietary levels of a number of ingredients. In the experiments, the results of which we are reporting at this time, we have incorporated varying amounts of methionine or cystine in the diet, replacing an equal amount of cornstarch, and have kept the other dietary constituents at constant levels. A supplement of thiamine, riboflavin, pyridoxine, pantothenic acid and nicotinic acid was given daily to each rat and with some of the diets we have further studied the effect of the inclusion of choline in the vitamin supplement. All of the rats received approximately 20% alcohol as a source of fluid. Similar results have been obtained in other experiments using water in place of alcohol.

A summary of the results of these studies is given in Table II. The figures are based on the results of histological examinations.

It is of interest that neither a high fat diet nor one containing free cystine is *essential* for the production of liver cirrhosis. Diet No. 542 contains 5% fat and 4% casein with no added amino acids and on this diet some rats developed cirrhosis of the liver.

Some rats on this same diet developed hemorrhage and necrosis of the liver. This necrosis appears to be identical with that seen by György and Golblatt.[1] It is primarily coagulative in type and centrolobular in location and is nearly always accompanied or

TABLE II.

Relation of Diet to Occurrence of Liver Hemorrhage and Necrosis and Cirrhosis.

Diet	No. of livers examined	No. with no cirrhosis and no hemorrhage and necrosis	Total No. with cirrhosis	Total No. with hemorrhage and necrosis	No. with hemorrhage and necrosis and cirrhosis
Basal diet (No. 542)*	16	10	3	6	3
0.04% added cystine	17	4	9	10	6
0.08% " "	9	0	9	2	2
0.16% " "	12	1	11	0	0
0.5% " " (Diet No. 545)	94	9	85	0	0
0.05% added methionine	27	14	5	11	3
0.2% " "	4	4	0	0	0
0.7% " "	10†	10	0	0	0
Choline chloride (20 mg per rat per day) given in supplement.					
0.04% added cystine	10	4	0	6	0
0.5% " "	15	12	2‡	1§	0

*Differs from diet No. 545 in that the 0.5% cystine is replaced by cornstarch.

†Including 8 rats which received both 0.7% methionine and 0.5% cystine.

‡One rat dying after 201 days on experiment and another dying after 245 days on experiment had slight liver cirrhosis.

§This rat died after 296 days on experiment.

even replaced by hemorrhage of quite variable extent in the necrotic areas. Sometimes only small periportal islets of surviving liver cells remain. When this picture and the hepatic cirrhosis described by us[6] occur together they appear to be essentially unrelated to each other, the hemorrhage and necrosis being superimposed on almost any phase in the development of the cirrhosis. Rarely is there any evidence of marginal organization about the hemorrhagic necrosis, and usually the hemorrhage is so fresh that no hemosiderin is demonstrable.

The inclusion of choline chloride in the vitamin supplement at a level of 20 mg per rat per day had a preventive effect on the development of the liver cirrhosis. None of the rats receiving diet No. 545 survived longer than 113 days unless choline was given. Among the rats on this diet receiving the 20 mg of choline chloride per rat per day there have been survivals up to one year; 2 of the 15 livers examined have shown slight cirrhosis after 201 and 245 days, respectively. These results do not exclude the possibility that a higher level of choline administration would completely prevent this liver cirrhosis, but neither do they exclude the possibility that some other factor is involved. Methionine, a choline precursor, had a preventive effect if given in sufficient quantity. Cystine, on the other hand, even in small amounts definitely increased the incidence of hepatic cirrhosis. A supplement of cystine in a choline-deficient diet is known to increase the deposition of liver fat in rats[10] while choline[11] and methionine[12] are known to exert a lipotropic effect; that these effects may have had an influence on the results of our experiments is indicated by our observation[6] that a fatty liver is one stage in the development of this type of hepatic cirrhosis.

While exerting a preventive effect on the development of this cirrhosis, choline had no such influence on the hemorrhage and necrosis. Six of 10 rats receiving choline and a diet containing 0.04% of added cystine showed hemorrhage and necrosis of the liver. Either methionine or cystine in sufficient amounts, however, has consistently prevented these lesions. The only exception encountered to date is one rat receiving 0.5% cystine which died after 296 days on experiment. The significance of this single exception cannot be stated at the present time. The prevention of these lesions by the sulfur-containing amino acids indicates that there may be an etiologic relationship between the hemorrhages in the livers of these

10 Beeston, A. W., and Channon, H. J., *Biochem. J.*, 1936, **30**, 280.
11 Best, C. H., Hershey, J. M., and Huntsman, M. E., *J. Physiol.*, 1932, **75**, 56.
12 Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

rats and the liver hemorrhage noted by Weichselbaum in rats with cystine deficiency[13] even though we have not observed the clinical symptoms which he described.

The observations recorded here do not indicate whether or not it is possible to vary the incidence of the hemorrhage and the incidence of the necrosis independently of each other.

The conclusions which seem to be justified by these experimental results are that *under these experimental conditions* (1) choline (and methionine, a choline precursor) have a preventive action on the development of this hepatic cirrhosis and (2) the sulfur-containing amino acids, cystine and methionine, have a preventive action on the development of the hepatic hemorrhage and necrosis. This suggests that the cirrhosis and the hemorrhage and necrosis are separate and distinct entities.

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Histochemical Studies of Phosphatase Distribution in Developing Teeth of Albino Rat.*

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The presence of phosphatase in teeth was first demonstrated by Robison and Soames[1] and confirmed by other studies made on extracts of the dental tissues.[2-7] It has been shown grossly that enzyme

13 Weichselbaum, T. D., *Quart. J. Exp. Physiol.*, 1935, **25**, 363.

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1 Robison, R., and Soames, K., *Biochem. J.*, 1924, **18**, 740.

2 Mackenzie, A. S., *Brit. Dent. J.*, 1933, **54**, 153.

3 Macfarlane, M. G., Patterson, L. M. B., and Robison, R., *Biochem. J.*, 1934, **28**, 720.

4 Provissionato, A., *La Stomatologia*, 1935, **33**, 619.

5 Berliner, F. S., *J. Dent. Res.*, 1936, **15**, 243.

6 Shafer, Z. M., *Northwestern U. Bull. of Dent. Res., Grad. Study Quart.*, 1937, **37**, 26.

7 Roche, J., and Bullinger, E., *Comptes Rendu Soc. de Biol.*, 1938, **129**, 976.

activity is higher in young growing teeth than in calcified adult teeth.[2, 7]

Gomori[8, 9] and Takamatsu[10] developed a technic for the histologic localization of phosphatase and reported on its distribution in various tissues. An illustration of a tooth germ of a mouse embryo showing widespread enzyme activity with no apparent specific localization was included in a study of normal and neoplastic tissues published by Kabat and Furth.[11]

The purpose of this paper is to report the results of qualitative histochemical studies made on the phosphatase distribution in developing teeth of young rats.

Material and Method. The studies were conducted on the developing molar and incisor teeth of 12 young white rats, one and 4 days old (Wistar stock). Immediately after the animals were killed the heads were halved in the mid-sagittal plane and fixed in 95% alcohol. The tissue was then dehydrated without decalcification, embedded in celloidin and sectioned at 12 to 14 μ. The sections were first stained for calcium and then incubated in a solution of calcium nitrate, β sodium glycerol phosphate and sodium barbital, following the technic of Gomori.[8] The tissues were then stained for precipitated phosphate, with the same technic and other modifications. A few sections were stained with hematoxylin and eosin after sectioning.

Controls were also studied by treating the dental tissues as follows: (1) Heating to 70°C for 15 minutes before incubating, to destroy

TABLE I.
Distribution of Phosphatase in Developing Tooth Germs of 1- and 4-day-old Rats.

	1 day			4 days		
	Incisor	First Molar	Second Molar	Incisor	First Molar	Second Molar
Enamel Organ						
Ameloblasts	—	—	—	—	—	—
Stratum Int.	+	+	—	+	+	+
Outer layers	+	—	—	+	Stellate reticulum + in some areas	—
Undifferentiated portion (odontogenic organ)	—	—	—	—	—	—
Pulp						
Odontoblasts	—	—	—	—	—	—
Tissue subjacent to odontoblasts	+	—	—	+	+	—

[8] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[9] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[10] Takamatsu, H., *Trans. Jap. Path. Soc.*, 1939, **29**, 492.

[11] Kabat, E. A., and Furth, L., *Am. J. Path.*, 1941, **17**, 303.

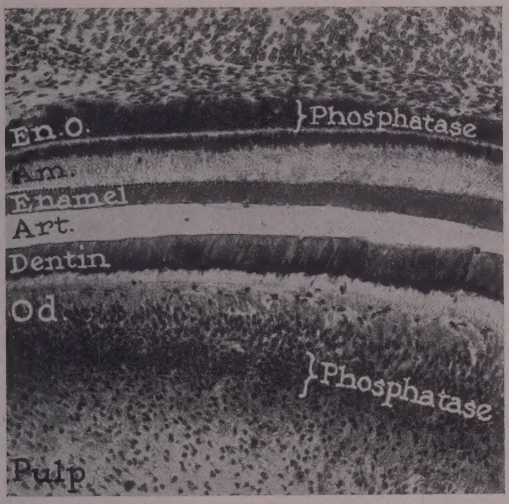

FIG. 1.

Photomicrograph of a longitudinal section from the mid-region of the labial of an incisor of a one-day-old rat. ×130. The tissue has been stained for phosphatase which appears as dark layers subjacent to the odontoblasts and ameloblasts. Am., ameloblasts; Art., artefact; En.O., fused layers of the stratum intermedium, stellate reticulum and outer enamel epithelium; Od., odontoblasts.

the enzyme; (2) incubation in distilled H_2O; (3) incubation in 2% sodium barbital, in 2% calcium nitrate, and in 2% sodium glycerol phosphate. The controls were then stained according to the Gomori technic.

Findings. All findings are summarized in the accompanying chart (Table I). All control sections were negative with respect to phosphatase.

The histology of the developing tooth germ is illustrated in Fig. 1, which shows the 4 cellular layers of the enamel organ, *i. e.*, the outer enamel epithelium and the stellate reticulum which are fused here, the stratum intermedium and the inner enamel epithelium or ameloblastic layer. The pulp with its connective tissue and odontoblastic layer is encased within the cap of calcified tissues (enamel and dentin).

One-day-old Rats. Molars. At one day the crowns of the first and second molars have attained their definitive pattern. The degree of differentiation of the ameloblasts and odontoblasts is graded from an undifferentiated cuboidal cell in the rapidly proliferating odontogenic area to a specialized columnar cell in the cusp region. The first molar is in a stage just prior to dentin formation. The phosphatase is localized in the stratum intermedium about the cusps.

There is no enzyme activity in the odontogenic area, the ameloblasts, odontoblasts or pulp cells.

Sections of the second molar show complete absence of the enzyme with the exception of a trace seen in a few cases in the stratum intermedium about the mesial cusp.

Incisors. (Fig. 1.) The gradation of cellular differentiation from basal to incisal is also noted in the incisor. At one day, calcification of the newly formed dentin is already proceeding, while the formation of the newly formed enamel has just begun. There is no evidence of phosphatase in the basal portion of the tooth (odontogenic epithelium). However, there is a high degree of enzyme activity in the stratum intermedium and in the continguous cells of the outer enamel epithelium anterior to this region. The pulpal tissue subjacent to the odontoblasts in the area of dentin calcification is a zone of high enzyme activity. The ameloblastic and odontoblastic layers are negative.†

Four-day-old Rats. Molars. Apposition of enamel and calcification of dentin are already proceeding in the first molar. There is a trace of uncalcified dentin in the second molar. This tooth is approximately at the same level of differentiation as the first molar of one-day-old rats.

The stratum intermedium about the crowns of first molars shows a high degree of enzyme activity. In addition, there is activity in some of the adjacent stellate reticulum, particularly about the cuspal-pit areas. In the pulp, those zones subjacent to the odontoblasts in the regions of dentin formation are positive.

The enzyme distribution in the second molar is similar to that found in the first molar of one-day-old rats.

Incisors. At 4 days the incisors have thicker layers of enamel and dentin. The phosphatase distribution is similar to that observed in the incisor of the one-day-old animal.

The problem of bone phosphatase was not investigated in this study. However, the connective tissue surrounding the crypt bone of the teeth showed extensive enzyme activity.

Discussion. Williams[12] expressed the belief that the stratum in-

† While the negative reaction of odontoblastic and ameloblastic layers was clearly evident, in some of the incisor sections a slight staining resembling a positive phosphatase reaction was noted. However, in view of the fact that this was found only occasionally and in a very irregular way, and never in the molars, there is good reason to believe that it was an artefact, *i.e.*, a result of a diffusion of the stain or of the overlapping of positive and negative layers rather than an indication of the actual presence of phosphatase.

12 Williams, J. L., *Dent. Cosmos*, 1896, **38**, 101, 453.

termedium played a rôle in the transfer of mineral salts from the blood to the enamel-forming cells. This study appears to support his view. The high phosphatase concentration in the stratum intermedium may accelerate the rate of hydrolysis of some tissue organic phosphates. This liberated inorganic phosphate may then be actively transported through the ameloblasts and precipitated in the enamel as the solubility product of the calcium salt (apatite) is exceeded. A similar relationship may exist between the pulpal cells and the odontoblasts. Undoubtedly the value of these findings would be enhanced if they could be supplemented by micro-incineration studies such as those of Hampp[13] in which the mineral distribution in the various layers of the tooth germ was demonstrated.

A correlation has been demonstrated between the degree of cellular organization and differentiation, and phosphatase activity in the developing tooth. This is of significance aside from its relation to the specific tissue; the tooth merely serves as an excellent test object for the study of phosphatase relations to the calcification process because in its development the calcification stages are sharply partitioned.

This absence of the enzyme from undifferentiated areas when calcification is not occurring and its appearance at levels of higher differentiation where calcification is about to occur (enamel) or is proceeding (dentin) would seem to emphasize the rôle of phosphatase in calcification.

Summary. 1. Phosphatase activity and localization in developing tooth germs of rats (1 and 4 days old) is correlated with the degree of cellular differentiation and the status of the calcification process. 2. The enzyme is shown to be localized (a) in the stratum intermedium, (b) in the outer enamel epithelium of the incisors, (c) to a lesser extent in the stellate reticulum about the first molar in the more advanced stages (4 days old), and (d) in the pulp contiguous to those odontoblasts in the regions of dentin calcification. 3. No phosphatase activity was demonstrated in the ameloblasts or odontoblasts. 4. The connective tissue surrounding the crypt bone showed extensive phosphatase activity.

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[13] Hampp, E. G., *Anat. Rec.*, 1940, **77**, 273.

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Relation of Cerebral Dysrhythmia to Eclampsia.*

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The etiology of eclampsia is unknown. Many aspects of the clinical picture of the eclamptic syndrome suggested the necessity for electroencephalographic studies. This report deals with the electroencephalographic findings and family histories of a group of 17 patients in whom a diagnosis of eclampsia was made because of convulsive seizures associated with the latter part of the puerperium.

These patients were studied from one week to 5 years after their hospital admissions. In one instance the tracing was taken one week after the eclampsia, in 2 cases the recording was made 10 days after the eclampsia, and in the remaining instances the electroencephalograms were made from 3 months to 5 years after the patients' admission for eclampsia. The intervals are adequate in most, if not in all instances, to exclude post-convulsive changes as a cause for the abnormal tracings.

The electroencephalographic technic consisted of 6 lead monopolar tracings, using the lobe of the ear as a reference point. The leads were taken from both frontal, parietal and occipital regions. Short periods of hyperventilation were included in each record. From the scalp electrodes the potentials were led off to Grass amplifiers and recorded on a 3-channel ink writer.

Preliminary analyses of the records, using the standards suggested by Gibbs,[1] Jasper,[2] Davis,[3] and others,[4] which include frequency, amplitude, wave form, and general stability, indicated that 13 out of 17 tracings were abnormal, *i. e.*, 77%. Two tracings were within normal limits and 2 were considered borderline.

Similar tracings were taken from 10 patients who had had mod-

*We are grateful for the coöperation of the Department of Obstetrics of Cincinnati General Hospital.

1 Gibbs, F. A., *J. Psychology*, 1937, **4**, 365.

2 Jasper, H., and Kershman, J., *Arch. Neurol. and Psych.*, 1941, **45**, 903.

3 Davis, P. A., *J. Neurophysiol.*, 1941, **4**, 92.

4 Gibbs, F. A., and Gibbs, E. L., *Atlas of Electroencephalography*, Lew A. Cummings Co., Cambridge, Mass., 1941.

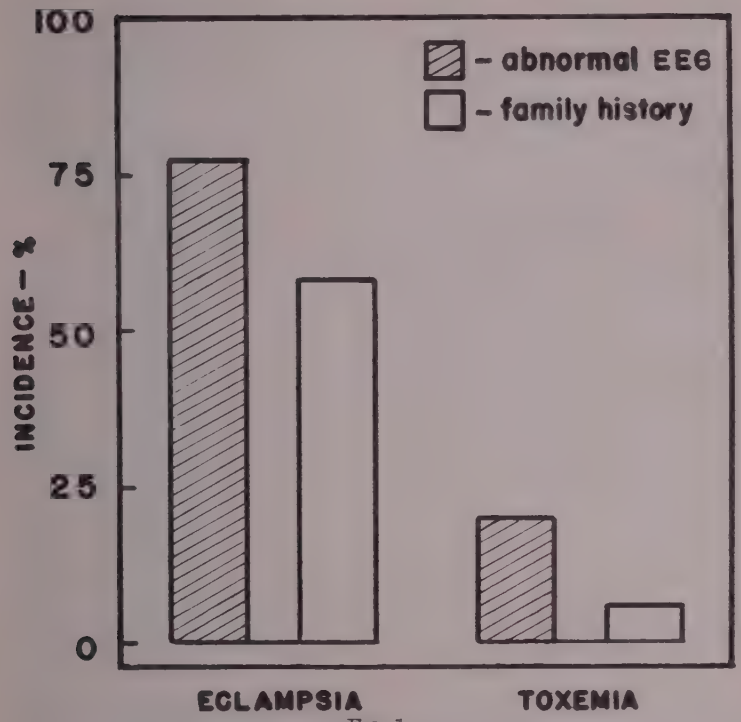

FIG. 1.

Illustrating the incidence of cerebral dysrhythmia and a family history of convulsive disorders in patients who have had eclampsia and toxemia of pregnancy.

erately severe to severe toxemia of pregnancy without convulsions (Toxemia—grade II). In this group there was only one record that was abnormal and 2 that were borderline or questionable.

A careful history taken on both groups of patients revealed that in 10 of 17 women with eclampsia there was a history of convulsive seizures either in the patient prior to her eclampsia or in some member of her immediate family. In only 2 patients was there a history of convulsive seizures prior to the pregnancy in which the diagnosis of eclampsia was made. In the group with toxemia alone there was only one instance with a family history of convulsions.

This preliminary study suggests that there may be a primary cerebral dysrhythmia present in those patients having the syndrome of eclampsia, and that the associated toxemia may be the "trigger mechanism" that exaggerates the inherent dysrhythmia to the degree that convulsions appear. In accord with this is the fact that many of the methods that have been successful in the therapy of eclampsia have depended upon the use of anticonvulsant measures.

The above data do not refer in any regard to the etiology of the toxemia, but do suggest that those patients who develop convulsions (eclampsia) in the course of their toxemia may have a primary cerebral dysrhythmia.

It may be inferred from the preceding that a careful history together with an electroencephalogram may be of great importance in determining those patients who may develop eclampsia. Furthermore, it is apparent that the proper therapy of eclampsia and pre-eclampsia may include those measures generally employed in the treatment of cerebral dysrhythmia. In accord with this, studies are now in progress to elucidate the action of dilantin and other anticonvulsants in the eclamptic and pre-eclamptic states.

Summary. Seventy-seven percent of 17 eclamptic women had electroencephalograms indicative of cerebral dysrhythmia. Fifty-eight percent of this series gave a family history of convulsive disorders.

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Effect of a Vacuum on Destruction of Bacteria by Germicides.

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The presence of a vacuum was found to have no effect on the destruction of bacteria by germicides. Regardless of whether the organisms were mixed with the germicidal dilutions and kept at atmospheric conditions or placed under a vacuum, the final results were the same.

Entirely different results were obtained, however, when gaseous germicides were employed. Tests carried out over a period of about 15 years have shown that the efficiency of the gases was greatly increased in the presence of a vacuum. In fact, under certain conditions organisms could not be destroyed unless a vacuum was employed.

Experimental. The gases which were tested included formaldehyde, methyl bromide, methyl formate, ethylene oxide, and carbon disulfide. With the exception of formaldehyde no one of the gases effected a sterilization after an exposure period of 2 hours. These non-germicidal gases are important for the destruction of insects

but are of very little, if any, value for the destruction of bacteria. Therefore, all tests were limited to the use of formaldehyde.

The organisms used included *Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Eberthella typhosa, Bacillus subtilis, Bacillus mycoides,* and *Bacillus anthracis.* Cultures incubated at 37° C for 72 hours were employed in the tests. In general, it was found that cultures increased in resistance up to 72 hours, after which the reverse effect occurred.

Two series of tests were performed. In one series a drop of culture was spread over the surface of small glass slides whereas in the other series a drop of culture was absorbed on short pieces of dental cotton rolls (Fig. 1). The glass or cotton preparations were used either immediately or dried for 3 hours at 37° C previous to treatment with formaldehyde.

The vacuum chamber used for the treatment of cultures is shown in Fig. 2. The door to the chamber comes in contact with a rubber gasket to produce a vacuum-tight tank. A small metal cylinder, tapered at the base and open at the top, is fastened to the inside of the door by means of clips, so that it may be easily removed for cleaning. This cylinder is placed directly below a cup situated above and outside of the sterilizer. A vacuum gauge is connected to the top of the chamber.

FIG. 1.

Tubes containing glass or cotton preparations of cultures used in the sterilizer tests.

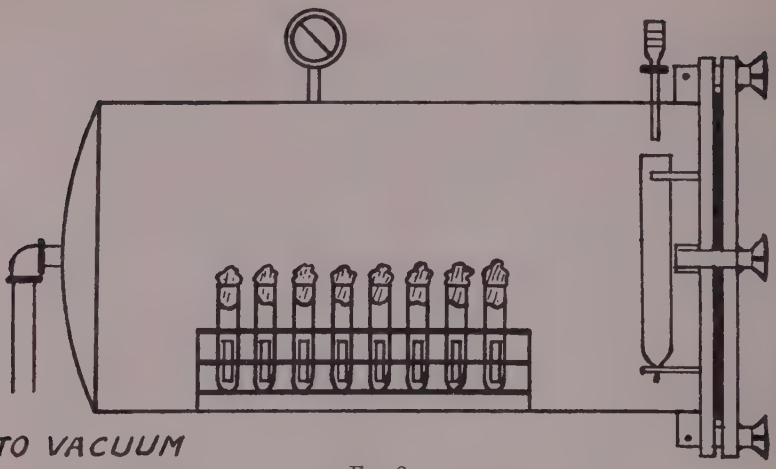

FIG. 2.
Vacuum sterilizer used for the treatment of the cultures.

The sterilizer is operated as follows: An appropriate amount of potassium permanganate in crystalline form is placed in the small cylinder fastened to the door. The cultures to be treated are placed inside of the sterilizer and the door tightly closed. The sterilizer is evacuated to 29 mercury inches (1 inch of pressure). A measured amount of solution of formaldehyde is pipetted into the cup on the top of the tank and allowed to slowly run into the metal cylinder fastened to the door. When the solution of formaldehyde comes in contact with the potassium permanganate, considerable heat is quickly generated volatilizing the formaldehyde gas. Sufficient solution is used to produce a drop of only one inch in the vacuum. Each cubic foot of sterilizer space required 6 cc of formaldehyde (commercial 37% solution) and 6 g of potassium permanganate. The sterilizer is held at 28 inches of vacuum throughout the entire exposure period. At the end of the exposure period, air is readmitted to the sterilizer and when the gauge reads zero the door can be opened.

The cultures were tested for sterility by dropping glass slides or cotton rolls into large test tubes containing 20 cc of nutrient broth. All tubes were incubated at 37°C for one month before making final readings.

Absence of a Vacuum. The first tests were performed by generating formaldehyde inside of the sterilizer, in the absence of a partial vacuum. The same amounts of formaldehyde and potassium permanganate were used as in the vacuum procedure. The periods of exposure to formaldehyde were for 1 hour and 2 hours. The cultures were absorbed on cotton rolls and dried for 3 hours previous

TABLE I.
No Vacuum.
One drop of a 72-hour culture of each organism was absorbed on cotton and dried 3 hours at 37°C previous to use.

Culture	Control tubes	Period of exposure to formaldehyde	
		1 hour	2 hours
Staph. aureus	+	+	+
Strep. faecalis	+	+	+
Ps. aeruginosa	+	+	—
E. coli	+	+	+
E. typhosa	+	+	—
B. subtilis	+	+	+
B. mycoides	+	+	+
B. anthracis	+	+	—

to use. The results are shown in Table I. It may be seen that an exposure period of 2 hours was not sufficient to effect a complete sterilization of all cultures. *Bacillus subtilis* showed greater resistance than any of the organisms employed in the tests. This organism was also more resistant to formaldehyde when exposed in a partial vacuum.

Presence of a Vacuum. Cultures on cotton were exposed to formaldehyde in the presence of 28 inches of vacuum for periods of 5 min 10 min and 25 min. The results are given in Table II. It may be seen that complete sterilization of all cultures was effected after an exposure period of 25 min. The results demonstrate the increased efficiency of formaldehyde in a vacuum as compared to its use under atmospheric conditions.

Cultures on glass slides were exposed to formaldehyde in the presence of 28 in. of vacuum for periods of 10 min., 25 min., 40 min., and 50 min. The results are also recorded in Table II. A

TABLE II.
Vacuum.
One drop of a 72-hour culture of each organism was absorbed on cotton or spread over the surface of glass slides and dried 3 hours at 37°C previous to use.

Culture	Control tubes	Period of exposure to formaldehyde						
		Cotton rolls			Glass preparations			
		5 min.	10 min.	25 min.	10 min.	25 min.	40 min.	50 min.
Staph. aureus	+	—	—	—	—	—	—	—
Strep. faecalis	+	+	+	—	—	—	—	—
Ps. aeruginosa	+	—	—	—	—	—	—	—
E. coli	+	—	—	—	—	—	—	—
E. typhosa	+	—	—	—	—	—	—	—
B. subtilis	+	+	+	—	+	+	+	—
B. mycoides	+	—	—	—	—	—	—	—
B. anthracis	+	—	—	—	—	—	—	—

period of 50 min. was required to destroy all of the organisms on glass slides whereas corresponding cultures on cotton were completely destroyed after an exposure of only 25 minutes.

Conclusions. It may be stated that the increased efficiency of formaldehyde as a germicide was not due to any effect of the vacuum on the gas but rather to the ability of the vacuum to increase the penetration of the gas. Formaldehyde is chiefly a surface disinfectant. The increased penetration in the presence of a vacuum made it possible for the gas to reach the organisms. The vacuum principle is capable of producing a very deep penetration of the gas into porous materials. The method is very efficient, cheap, easily performed, and indispensable for the sterilization of objects likely to be injured by the application of dry or moist heat.

13686 P

Reactions of 2-Methyl-1, 4-Naphthoquinone (Menadione) with Whole Blood and Plasma *in vitro*.

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Recently Seligman and coworkers[1] reported that vitamin K_1 has such a persistent and prolonged action that a single small dose is adequate for the treatment of even severe cases of hypoprothrombinemia, and may well do the work of repeated doses of other agents. This point has been emphasized by Fieser.[2] In the course of our work on the development of an oxidation-reduction method for the determination of vitamin K_1[3] it was found that the vitamin is remarkably stable in whole blood, but menadione is rapidly destroyed, and further menadione, unlike the vitamin, causes a marked methemoglobin formation.

Experimental. An oxalated sample of freshly drawn dog blood was divided into three 30 cc samples. 1.5 mg of 2-methyl-1,4-naphthoquinone, an equivalent weight of 2,3-dimethyl-1,4-naphtho-

1 Seligman, A. M., Hurwitz, A., Frank, H. A., and Davis, W. A., *Surg. Gynecol. Obstet.*, 1941, **78**, 686.

2 Fieser, L. F., *Ann. Int. Med.*, 1941, **15**, 648.

3 Scudi, J. V., and Buhs, R. P., *J. Biol. Chem.*, 1941, **141**, 451. (See also paper in press.)

quinone, or an equivalent weight of vitamin K_1, each dissolved in 0.5 cc of ether was added to the blood samples. As with other quinones,[4] when the samples were mixed, the blood containing the menadione rapidly darkened in color, and after 15 to 20 minutes 50% of the blood pigment was found, spectroscopically, to be present as methemoglobin. Under these conditions neither vitamin K_1 nor 2,3-dimethyl-1,4-naphthoquinone caused a methemoglobincythemia, possibly because of cell impermeability.[5]

Twenty minutes after the preparation of the blood samples, 5 cc aliquots were added dropwise to 70 cc of boiling absolute alcohol. The mixtures were cooled; 40 cc of absolute ether was added, and the mixtures were shaken in the absence of light. Volumes were adjusted to 150 cc with alcohol and the samples were filtered. Aliquots of the filtrates were concentrated under nitrogen *in vacuo* and butanol was added to transfer the quinones to butanol without evaporation to dryness. The butanol solutions were then analyzed as previously described.[3] Pure solutions of menadione gave 100% recoveries. In the experiments with blood, quantitative recoveries of the added vitamin K_1, and the 2,3-dimethyl-1,4-naphthoquinone were obtained. The analytical method is capable of detecting 1 γ per cc of solution, and the concentrates analyzed should have contained 10 to 20 γ per cc, but no menadione was found. Nor were recoveries effected following peptic hydrolysis of the blood proteins even though a variety of extraction procedures were tried.

Similar experiments performed with fresh oxalated plasma gave 40 to 50% recoveries of added menadione, while 1- and 2-day-old samples of plasma gave recoveries of 60 to 70%. The menadione caused the plasma samples to acquire a deep yellow color upon standing in the absence of light.

Summary. When added to whole, unlaked blood, menadione causes a marked methemoglobin formation, and the drug is rapidly converted to some other substance (or substances). Assays with vitamin K-deficient chicks showed that these reactions cause a marked loss in the antihemorrhagic activity of the menadione. Vitamin K_1, unlike menadione, does not produce methemoglobin under comparable conditions, and the vitamin is remarkably stable in whole blood.

4 Austin, J. H., and Drabkin, D. L., *J. Biol. Chem.*, 1935, **112**, 67.

5 Van Slyke, D. D., and Vollmund, E., *J. Biol. Chem.*, 1925, **66**, 415.

13687

Dextrose Administration and Vitamins B.*

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Several components of the vitamin B complex appear to be necessary parts of enzyme-coenzyme systems of tissue oxidation. In the oxidation or dismutation of pyruvic acid by animal tissue, thiamine, riboflavin and nicotinic acid appear to be necessary.[1] Abderhalden and Wertheimer[2] found that pigeons in B_1 avitaminosis had glycogen accumulation in the liver and heart which increased as the vitamin deficiency progressed and which decreased when thiamine was given. Westenbrink[3] concluded that carbohydrate in the diet hastens the appearance of symptoms of thiamine deficiency in pigeons and that fat in the diet delays symptoms. He expressed the opinion that the symptoms were largely due to the accumulation of toxic products of improper carbohydrate metabolism. Platt and Lu[4] found that the pyruvic acid content of blood, urine and spinal fluid was increased in thiamine deficiency.

Helmholz and Bollman[5] found that intravenous injection of large amounts of dextrose in rabbits was more toxic and produced less diuresis than equivalent amounts of sucrose. The obvious conclusion was drawn that the toxic effects of excessive dextrose were associated with the metabolism of this substance, since sucrose is excreted unchanged. It may be possible that some of the toxic effects of excessive dextrose under these conditions are due to the rapid depletion of some of the enzyme-vitamin system involved in the oxidation of dextrose and its intermediary products. Several experiments in which large amounts of dextrose were administered intravenously to rabbits failed to show any advantage in the simultaneous administration of large amounts of thiamine, riboflavin, nicotinamide, pyridoxine and pantothenic acid. These animals had not been depleted

* Read before the meeting of the Federation of American Societies for Experimental Biology, Boston, Massachusetts, March 31 to April 4, 1942.

1 Lipton, M. A., and Elvehjem, C. A., *Cold Spring Harbor Symp. Quart. Biol.*, 1939, **7**, 184; Lipmann, F., *ibid.*, 1939, **7**, 248.

2 Abderhalden, Emil, and Wertheimer, Ernst, *Arch. f. d. ges. Physiol.*, 1932, **230**, 601.

3 Westenbrink, H. J. K., *Arch. néerl. de physiol.*, 1935, **20**, 481.

4 Platt, B. S., and Lu, G. D., *Quart. J. Med.*, 1936, **5**, 355.

5 Helmholz, H. F., and Bollman, J. L., *J. Lab. and Clin. Med.*, 1940, **25**, 1180.

previously of vitamins and no deficiency was present at the beginning of the injection of dextrose.

Another attempt was made to indicate some difference in the utilization of large amounts of dextrose by vitamin B-deficient rats. Young rats were fed a vitamin B-deficient diet for 3 weeks and then given large amounts of dextrose as frequently as possible by stomach tube. Some rats received in addition large amounts of the B vitamins. Both groups of rats survived only 2 or 3 days under this regimen and no advantage was clear in either group.

A third group of experiments definitely indicated that vitamin deficient rats are better able to metabolize dextrose and survive protein deficiency when thiamine is added than when it is not. Rats previously deprived of vitamin B survived on a diet of dextrose from 2 to 4 times longer when thiamine was given than when it was not. Other components of the vitamin B complex, riboflavin, pyridoxine, pantothenic acid and choline appeared to be ineffective. Nicotinamide appeared to have some slight effect.

Experimental Procedure. Young rats of the Wistar strain which had been receiving a diet of Purina fox chow were placed on the following diet: casein, 10; sucrose, 80; butter, 4; salt mixture, Steenbock's No. 40, 4; powdered agar, 2 and cod liver oil concentrate, 0.015. The rats gained weight for several days but soon began to lose weight so that after 2 weeks they weighed the same or less than their original weight. Each rat was kept in an individual cage and any that did not continue to eat some of the diet each day were not used. Rats of 65 to 90 g body weight were used, and 12 were selected for each group. At least 10 of these continued throughout the experiment. Each group was selected so that the range of weight variation was small in each group. Such selection was necessary because the larger animals lost less weight and survived the diet better than the smaller ones. The experiments reported in this series were done after the rats had been on the vitamin B-free diet for 14 to 21 days. Dextrose was then substituted for the vitamin B-free diet and in addition each animal received by stomach tube 4 cc of 25% solution of dextrose twice daily.

The vitamins administered were injected subcutaneously in aqueous solution (0.1 cc) once daily during the time the animals received only glucose. Thiamine hydrochloride, pyridoxine hydrochloride and pantothenic acid were each given in amounts of 0.1 mg daily; riboflavin, 0.03 mg; nicotinamide, 1.0 mg, and choline chloride, 5.0 mg.

Results. With the vitamin B-free diet employed, young rats

(65 to 90 g) were found to survive for 23 to 45 days. Lack of appetite and undernutrition may be a great factor in the survival of the vitamin deficient animals. After 3 weeks of vitamin depletion these rats, continued on the vitamin-free diet, were restored by the subcutaneous injection, 3 times each week, of thiamine, riboflavin, nicotinamide, pyridoxine hydrochloride and pantothenic acid in the amounts given previously. These animals rapidly gained weight and appeared in good condition for 3 months, when the experiment was terminated.

After 14 days or more of vitamin depletion the rats survived fasting only 2 or 3 days. Substitution of dextrose for the diet and administration of dextrose by stomach tube enabled the rats to live considerably longer than the fasted rats (Table I). The length of life of the dextrose-fed rats was somewhat shorter than that of animals continued on the vitamin-free diet.

Table II lists the groups of rats which received dextrose and subcutaneous injections of vitamins. The average length of life for

TABLE I.
Rats Receiving Dextrose without Supplements.

Days receiving vit. B free diet	Avg wt, g	Lived,* days
14	66	7 (5-9)
16	69	11 (9-14)
18	71	16 (12-20)
21	74	11 (7-14)

*First figure is average survival; figures in parentheses indicate the shortest and the longest survival.

TABLE II.
Rats Receiving Dextrose and Vitamins.

Days receiving vit. B free diet	Avg wt, g	Lived,* days	Comparison†	Vitamins injected
14	67	26 (13-39)	372	Thiamine, riboflavin, nicotinamide, pyridoxine, pantothenic acid
16	67	27 (24-33)	245	
16	73	26 (18-32)	236	Thiamine, riboflavin, nicotinamide, pyridoxine, pantothenic acid, choline
18	74	32 (21-41)	200	Thiamine
21	74	41 (28-65)	372	Thiamine
18	77	25 (18-35)	156	Nicotinamide
18	69	17 (14-22)	106	Riboflavin
16	65	8 (5-13)	73	Choline
16	68	10 (7-14)	91	Pyridoxine
16	70	12 (8-15)	109	Pantothenic acid

*First figure is average survival; figures in parentheses indicate the shortest and the longest survival.

†Life compared with that of rats without supplement, per cent.

each group is given and the figures in parenthesis indicate the shortest and the longest survival. The length of life is compared with that of the rats receiving dextrose without vitamins. This comparison is made with the comparable control group of rats receiving only dextrose because of the variation in the different groups of control animals. It is obvious that the injection of the mixed vitamins definitely prolonged the life of the dextrose-fed rats. Thiamine alone had a similar effect, but riboflavin, choline, pyridoxine and pantothenic acid were without effect. Some prolongation of life was present in the rats receiving nicotinamide.

Rats not previously fed a vitamin deficient diet survived 25 days when receiving only dextrose in the diet and by stomach tube. The subcutaneous injection of thiamine did not significantly prolong the life of a similar group of animals receiving only dextrose (Table III).

Comment. In these experiments the survival of vitamin B-deficient rats receiving dextrose was slightly shorter than that of similar animals continued on the vitamin-free diet containing protein. The difference in survival time could be lengthened somewhat by administration of the vitamin B-free diet by stomach tube. Any shortening of the time of survival due to dextrose feeding seems to be referable to protein deficiency rather than to the thiamine deficiency. The slight difference in the survival time of the rats, not previously depleted of vitamins, which received dextrose alone and those which received dextrose and thiamine, also indicates that the protein deficiency is the major factor. Since the rats were receiving a diet rich in carbohydrate prior to the substitution of dextrose alone, one should not expect the relatively small increase of carbohydrate consumption to influence materially the time of onset of symptoms of thiamine deficiency.

The rats which received thiamine and dextrose after previous depletion of vitamin B survived definitely longer than similar animals not receiving thiamine. Also it should be noted that the vitamin B-depleted rats receiving dextrose alone did not survive as long as rats not previously depleted and receiving dextrose only. Thiamine enables rats to survive longer on a diet of dextrose (and also devoid

TABLE III.
Rats without Previous Vitamin Depletion Receiving Dextrose.

Avg wt, g	Lived,* days	Wt loss, g	Additional treatment
77	25 (19-29)	30	None
82	27 (20-39)	35	0.1 mg thiamine daily

*First figure is average survival; figures in parentheses indicate the shortest and the longest survival.

of protein and fat) than rats that do not receive thiamine. Unfortunately I have no direct data concerning any possible protein sparing action of thiamine in addition to that of the dextrose given. The loss of weight of both groups of animals was very similar, being rapid at first and slowing as time continued. At the time of death of the dextrose-fed rats the weight of those receiving thiamine was similar. With the further survival of the thiamine-treated rats loss of weight continued so that they weighed less than the dextrose rats at the time of death. The amount of dextrose consumed each day was similar in both groups of animals.

Summary. Young rats weighing 65 to 90 g were maintained for 14 to 21 days on a vitamin B-free diet (casein 10, sucrose 80, butter 4, salts, agar and vitamins A and D). At this time these animals survived fasting but 2 or 3 days. Substitution of a diet of dextrose, supplemented by dextrose solution by stomach tube, permitted survival of from 7 to 16 days.

Vitamin-depleted rats receiving dextrose and thiamine survived 2 to 4 times as long as those receiving only dextrose. Nicotinamide appeared to have some effect, but riboflavin, pyridoxine hydrochloride, pantothenic acid and choline had no appreciable effect in prolonging the life of the dextrose-fed rats. All of the foregoing vitamins given together had no further beneficial effect than when thiamine alone was given.

When dextrose was substituted immediately following an adequate vitamin-containing diet, rats survived 19 to 29 days. The injection of thiamine hydrochloride or the vitamin mixture did not appreciably alter the survival time of these animals.

13688 P

A Difference in Metabolic Requirements of Meningococcus and Gonococcus.*

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Gordon and McLeod[1] found that the gonococcus does not grow on a medium based on tryptic digest of casein and gave this point con-

* Aided by grants from the A. B. Kuppenheimer Fund and the U. S. Public Health Service.

1 Gordon, J., and McLeod, J. W., *J. Path. and Bact.*, 1926, **29**, 13.

siderable study. They came to the conclusion that this lack of growth is due to inhibition by large amount of amino acid, and, especially, the high tryptophane content of the digested casein. McLeod[2] and, recently, Mueller and coworkers[3] have reported finding that cystine in high concentrations produces constant, marked, inhibitory effects to growth of this Neisseria. McLeod went further to suggest that cystine *may* favor growth of certain strains of gonococcus, especially when the medium contains considerable blood. Higginbotham[4] has described the use of cystine in chocolate agar medium for growth of gonococcus in specimens shipped to the laboratory for examination. Kobayashi and Nishikawa[5] have said that the growth of gonococcus, meningococcus, streptococcus and pneumococcus on a medium containing beef extract, serum, agar, etc., is favored by 0.5% to 0.005% cystine.

We require rather large quantities of gonococcus and meningococcus cells to carry on our comparative study of the chemical nature of these bacteria. Our stock strains grow very well on a medium[6, 7] containing tryptic digest or acid hydrolysate of egg white, buffer, dextrose and agar.

In recent months, however, casein was substituted for egg white. It was found that none of the gonococcus strains available will grow on this medium although the meningococcus types I, II and atypical variations thereof grow very well. Pure casein contains much less cystine than egg white, although the methionine contents of these 2 proteins are not far different; furthermore, it has been stated[8] that cystine is destroyed when liberated from casein by tryptic digestion as it is usually carried on in the alkaline condition. Analysis by Sullivan's[9] procedure showed none of this amino acid in our product of tryptic digestion. An addition of about 0.05% cystine, however, improves the casein digest medium, with respect to gonococcus growth, to a par with the egg white digest medium. The growth of meningococcus is not improved perceptibly by this altera-

2 McLeod, J. W., *Brit. J. Exp. Path.*, 1927, **8**, 25.

3 Mueller, J. Howard, Hinton, Jace., and Miller, Pauline A., *J. Bact.*, 1942, **43**, 100.

4 Higginbotham, Margaret, personal communication to the author.

5 Kobayashi, B., and Nishikawa, E., *Japan Med. World*, 1921, **1**, 11.

6 Miller, C. Phillip, and Castles, Ruth, PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 123.

7 Boor, Alden K., and Miller, C. Phillip, PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 370.

8 Jones, D. Breese, and Gersdorff, Charles E. F., *J. Biol. Chem.*, 1939, **129**, 207.

9 Sullivan, M. X., Supplement No. 78 to U. S. Public Health Service Reports, pages 1-13, 1929.

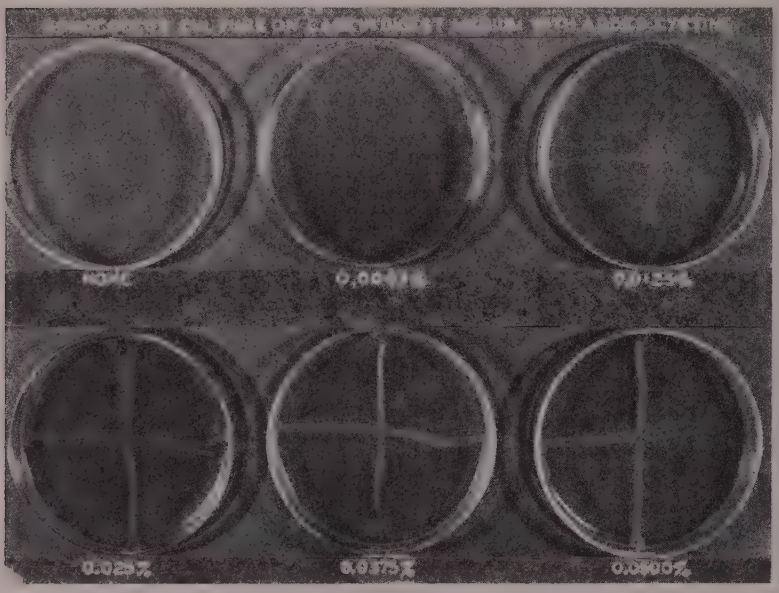

Fig. 1.
Gonococcus suspension applied with swab equally to each of the six plates. Sixteen-hour culture.

tion. The addition of cystine to the medium prepared from tryptic digest of egg white does not induce a larger yield of gonococci, nor meningococci.

A very slight growth of our stock strains of gonococcus can be detected on that medium containing as little as 0.00125% cystine, but the best growth develops with 0.0250% to 0.075% of this added amino acid. With increases in cystine above 0.10% a decrease in growth ensues, but with cystine present even to the point of saturation, some growth develops and those colonies which do multiply, do so very rapidly and after a few days are as heavy as the cultures on the media with the lesser, optimal, amounts of cystine.

These gonococcus cultures allowed to stand on the medium still contained viable organisms after 10 days, although the bacteria were not alive after 14 days. A meningococcus strain is still alive and growing after being on this semisolid medium in the incubator for more than a month.

The use of cystine by the gonococcus is evidently something more than as a "building stone" in cellular protein formation. An analysis[9] of the gonococcus cells grown on this medium showed only a trace of cystine, not more than was found in meningococcus cellular sub-

stance. It is an interesting observation in comparative cellular requirements that methionine does not replace cystine in the stimulation of gonococcus growth. This cystine requirement of the gonococcus was found to be only a type requirement; certain other substances containing sulfur, and potentially -SH formers, satisfactorily replaced this amino acid. Among these were cysteine hydrochloride, glutathione, thioglycolic acid, flowers of sulfur and barium sulfide.

Summary. A rapid and heavy growth of meningococcus developed on an agar medium containing tryptic digest of casein, dextrose and buffer at pH 7.4-7.6. However, this medium failed to support growth of the gonococcus. Addition to the medium of a small amount of cystine, however, stimulated growth of this organism. The growth of gonococcus on this casein digest medium, with cystine, was as heavy as that of meningococcus. The growth of meningococcus did not seem to be improved by this addition of cystine to the medium.

13689

Susceptibility to Convulsions in Relation to Age. II. Influence of Bile in Rats.

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In a previous communication a hypothesis was developed that the high susceptibility to convulsions of infants and young children may be due to the action of irritants entering the brain in the absence of a hypothetical hematoencephalic barrier.[1] Thus, it was observed that acid fuchsin, a water soluble neurophilic, sulfonated dye, will produce convulsions in rats of from 17 to 20 days of age and not thereafter. This phenomenon may be associated with the ability of acid fuchsin to penetrate the brain of the young animals and not of adults.

In the attempt to obtain further information concerning this peculiarity of the young brain, the influence of bile was studied. It has been known for some time that the brains of young children dying with jaundice may be icteric, while those of severely jaundiced adults are not.[2] Also, it has been observed that jaundiced infants

[1] Frohlich, A., and Mirsky, I. A., *Arch. Neur. and Psych.*, 1942, **47**, 30.

[2] Orth, J., *Virch. Arch. f. Pathol. Anatomie*, 1875, **63**, 447.

TABLE I.
Susceptibility to Convulsions in Different Age Groups after Administration of Bile.*

Age	No.	Dose mg/g	No. with convulsions	% dead
1-5	37	0.1-0.5	34	12
6-10	16	0.1-0.5	13	6
11-15	11	0.3-1.0	0	0
16-adult	22	0.5-1.0	0	0

*Parke Davis & Co. "Concentrated Bile."

may develop convulsions.[3] These factors seemed to warrant an attempt at experimental elucidation.

Experimental. Groups of rats of both sexes which ranged in age from 1 day to over 100 days were tested with various doses of bile (prepared from Parke Davis & Co. "Concentrated Bile"). Table I indicates the number of animals employed in each age group. The bile was administered intraperitoneally in a 1% solution, and the dosage varied from 0.1 to 1 mg of bile per gram of body weight. No irritation was apparent as a consequence of the injection of bile.

The incidence of convulsions is detailed in Table I and illustrated in Fig. 1. Within 100 minutes after the intraperitoneal injection of bile, convulsions occurred in 92% of rats between the ages of 1 and 5 days, and in 81% of rats between the ages of 6 and 10 days. These convulsions would last several hours and in a few instances terminated with the death of the animal. The convulsions were gradual in onset. The early abnormal signs were an increase of reflex activity, and much "wriggling" of the animal. This was followed by rhythmic clonic seizures during which the animal shrieked. Tonic contractions of the abdominal wall occurred frequently. Consequent to the full development of the convulsions the animals became somewhat lethargic and would remain in one position for several hours. They were then returned to their mothers and when examined 18 hours later no abnormality could be observed.

The intraperitoneal administration of bile to animals older than 10 days of age caused no apparent disturbance. However, when 1 mg of bile in a 1% solution was introduced directly into the brain by way of a trephine opening, clonic convulsions began within 10 minutes. Histological examination of the brains of bile-treated animals up to 10 days of age occasionally revealed the presence of pigment in various parts of the brain within two hours of the injection. The pigment was observed in greatest concentration in the

[3] Zimmerman, H. M., and Yannet, H., *Am. J. Dis. Child.*, 1933, **45**, 745.

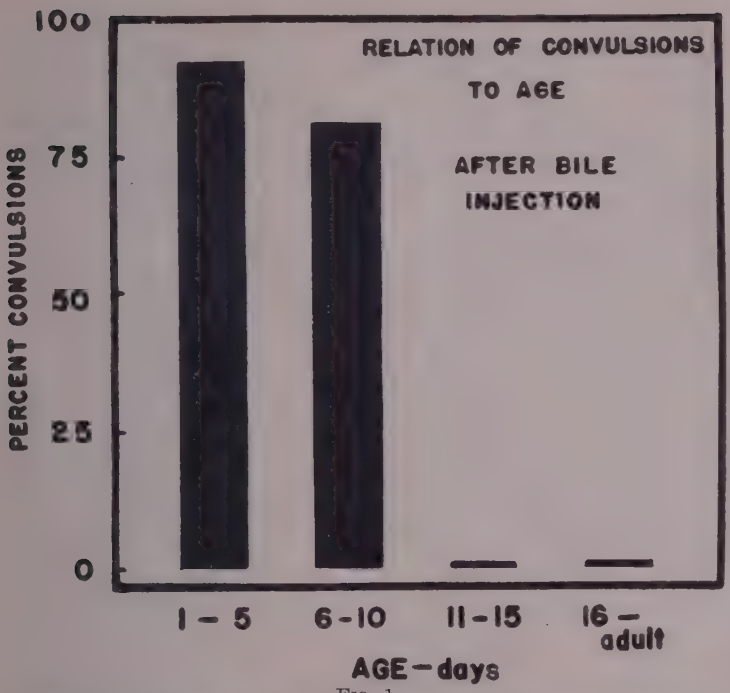

FIG. 1.

Illustrating the relation of age to the development of convulsions consequent to the injection of bile.

periventricular areas of the cornu ammonis.* Examination of the brains of the adult animals after the intraperitoneal injection of bile revealed no pigment.

The syndrome of "Kernicterus" has been known for some time[2] but has received relatively little attention. In the majority of such cases reported in the literature biliary pigment was found in the interstitial tissue of the cornu ammonis, in the cerebral cortex and in the cells of the basal ganglia. Zimmerman and Yannet[3] reported that convulsions or spasticity occurred in more than 40% of such cases. Other evidences of cerebral involvement were observed in many of the other cases.

Our studies with rats support the hypothesis that a hematoencephalic barrier is present in older animals and not in the very young. The reason for the cessation of response to bile in rats at 10 days of age and to acid fuchsin at 17 to 20 days of age is not clear.

* We are indebted to Dr. I. Mark Sheinker of the Laboratory of Neuropathology, University of Cincinnati College of Medicine, for performing the histological studies.

These considerations suggest that the increased susceptibility of infants to convulsions is associated with the ease with which various irritants can enter the brain tissue. However, another factor also must be considered. It is probable that not only is it much easier for various substances to enter the central nervous system of the young, but that this organ is much more sensitive to irritants. Thus, Brill and Seidemann[4] have observed that the electroencephalograms of normal children show a considerable tendency to dysrhythmia during hyperventilation and that this tendency diminishes with advancing age. It is probable that in the presence of increased tissue susceptibility, as indicated by cerebral dysrhythmia, and in the absence of a hematoencephalic barrier, as indicated by the passage of bile and dyes into the brain, a toxic agent will produce seizures in young subjects when it would be ineffective in older ones.

Summary. The intraperitoneal injection of bile results in the production of convulsive seizures in rats up to 10 days of age and not thereafter. The intracerebral injection of bile produces convulsions in adult rats. The hypothesis is developed that young animals are more susceptible to convulsive seizures because of an inherent cerebral dysrhythmia and a deficient hematoencephalic barrier.

13690

Subarachnoid Injection of Thiamine in Cats; Unmasking of Brain Lesions by Induced Thiamine Deficiency.

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This work was undertaken to determine whether the neurological symptoms of thiamine deficiency could be treated more effectively by introduction of the vitamin into the subarachnoid space than by the usual routes of administration. It seemed possible that thiamine might be held up or delayed at the hemato-encephalic barrier when given by the usual routes. Stern[1] has made a rather exuberant report of the effectiveness of subarachnoid injection of thiamine in patients with a variety of diseases.

[4] Brill, N. Q., and Seidemann, H., *Am. J. Psych.*, 1941, **98**, 250.

[1] Stern, E. L., *Am. J. Surg.*, 1938, **39**, 495.

Cats were selected because of their suitability for cisternal puncture. Fifteen adult animals were placed on the diet used by Cowgill[2] for dogs. Amount of food consumed and weight of each animal were recorded daily. Appetite diminished markedly within a few days and tube feeding was adopted to prevent nutritional disturbances other than thiamine deficiency. All the animals developed typical symptoms beginning with loss of appetite and progressing to weakness, apathy, vomiting, incoördination, and in some cases to convulsions and paralysis. Severe and prostrating symptoms were apt to develop suddenly between the third and fourth weeks, animals becoming almost moribund overnight. One animal was kept on the above diet for 70 days and injected twice weekly with 1.0 mg thiamine chloride. No symptoms of thiamine deficiency appeared.

Several hours to several days after onset of severe symptoms thiamine chloride was administered, 0.5 to 2.0 mg in aqueous solution or in normal saline. Injection was made either intravenously, intramuscularly or into the cisterna magna after removal of an equivalent amount of C.S.F. The animals had reached such a stage of thiamine depletion that no anesthetic was necessary during cisternal puncture. Rate of recovery from symptoms was noted in each instance.

Animals undergoing thiamine depletion for the first time showed rapid and spectacular recovery after a single injection of thiamine. Gait and strength were almost normal within one hour and completely normal by the following day. Recovery was equally good when the vitamin was given intravenously or intramuscularly as when injection was made into the subarachnoid space.

Six recovered animals were kept on the diet and within 14 to 20 days showed symptoms of thiamine deficiency again. The above therapeutic procedures were repeated but improvement occurred much more slowly and some residues of weakness and incoördination usually remained despite repeated daily injections of thiamine. Four animals died despite thiamine administration. In 2 instances thiamine depletion was allowed to occur for a third time. Both animals died after onset of deficiency symptoms despite large amounts of thiamine.

Following subarachnoid injection of thiamine chloride some degree of meningeal irritation occurred as evidenced by increased cell count in the C.S.F. In order to check this point cisternal injection of thiamine chloride was performed 21 times in normal cats. Cell counts were made on the C.S.F. before injection and at 12 and 24 hours after the procedure. Pleocytosis was noted in all instances after

[2] Cowgill, G. R., *Am. J. Physiol.*, 1921, **57**, 420.

injection. The lowest count recorded after 24 hours was 75 lymphocytes per cu mm and the highest was 1500 per cu mm. Four different commercial preparations of thiamine chloride from ampules were used, also a sterile aqueous solution prepared by us from the crystalline substance. Acidity of the various preparations varied somewhat and reactions were more marked with the more acid preparations. Reactions occurred, however, even with neutralized preparations.

We have observed signs of meningeal irritation in 4 patients to whom thiamine chloride was administered by the lumbar route. Increasing doses of from 1.0 to 12.0 mg were injected at intervals of several days. The preparations were so faintly acid that they were neutralized by only 2 to 6 cc of C.S.F. When doses of 6.0 to 8.0 mg were reached the patients complained of some stiffness in the back and pain down the legs, whilst protein and cells in the C.S.F. were found to be elevated (Fig. 1). No serious or persisting results of subarachnoid injection of thiamine were noted either in animals or humans.

Four cats with surgical brain scars were placed on the thiamine deficient diet. They had been operated upon several months previously by our colleague, Dr. T. C. Erickson. They showed no clinical evidence of neurological defect. The first symptoms of deficiency were referable to the area of brain damage and appeared to be unmasked by the vitamin deficiency. The most striking result was seen in a cat from which the left frontal pole had been removed.

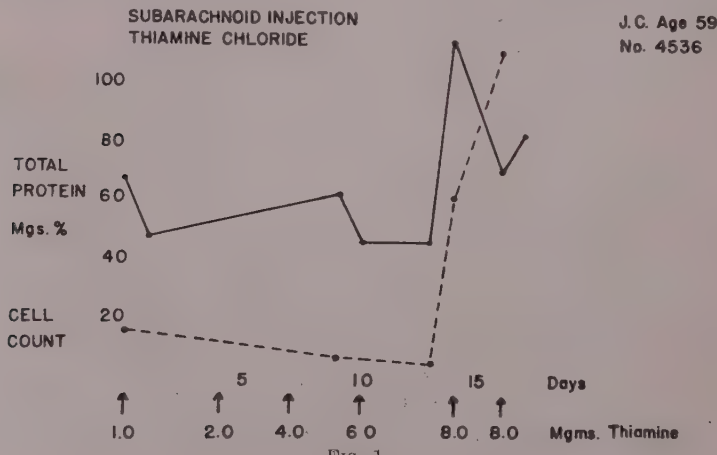

FIG. 1.

After 28 days on the diet the limbs on right side of the body became spastic and extended. Later a right sided convulsive seizure occurred with head turned to right and right limbs rigid. One hour after intracisternal injection of thiamine all symptoms had disappeared. The diet was continued and 15 days later the right limbs were again spastic and frequent tonic convulsions involved the right side. Convulsions ceased after administration of 0.5 mg thiamine but a residue of spastic paralysis remained in the right hind limb. The animal died 17 days later whilst in thiamine deficiency for the third time.

The above findings may explain partial improvement with thiamine therapy of some neurological diseases which are not the direct result of vitamin deficiency.

Summary. 1. Subarachnoid injection of thiamine causes rapid recovery of thiamine-deficient cats. Recovery is equally rapid when the vitamin is given intravenously or intramuscularly. 2. Moderate signs of meningeal irritation follow introduction of thiamine chloride into the subarachnoid space. 3. Thiamine deficiency may unmask symptoms due to an organic defect in the brain.

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Effects of Cozymase upon Growth of Staphylococci and Antistaphylococcal Action of Sulfonamide Compounds.*

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Following the report of Knight[1] that thiamin chloride and nicotinic acid are essential growth factors for *S. aureus*, Gladstone[2] demonstrated that a medium of known chemical composition may be used for growth experiments with staphylococci. A chemically-defined medium has certain advantages for the study of the bacteriostatic effect of the sulfonamide compounds upon staphylococci.[3] West and Coburn[4] utilized Knight's "synthetic" medium for an investigation

* Aided by grants from the Committee on Scientific Research of the American Medical Association, and the Graduate School of the University of Minnesota.

1 Knight, B. C. J. G., *Brit. J. Exp. Path.*, 1935, **16**, 315.

2 Gladstone, G. P., *Brit. J. Exp. Path.*, 1937, **18**, 322.

3 Spink, W. W., and Jermsta, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 395.

4 West, R., and Coburn, A. F., *J. Exp. Med.*, 1940, **72**, 91.

of the bacteriostatic effect of sulfapyridine upon the staphylococcus. They concluded that cozymase (Coenzyme I) interfered with the antistaphylococcal action of sulfapyridine, whereas nicotinic acid did not. Strauss, Dingle and Finland[5] were unable to confirm this finding. They made the interesting observation that cozymase could replace the essential growth factors, nicotinic acid and thiamin chloride, and suggested that impurities in the cozymase were responsible for this finding.

Because of the divergence of reported results concerning the effect of cozymase upon the bacteriostatic action of the sulfonamide compounds for the staphylococcus, we are recording our observations. An explanation is also presented for the confirmed observation that cozymase will stimulate growth of the staphylococcus in the presumed absence of nicotinic acid and thiamin chloride.

Material and Methods. The previously mentioned investigators used either a casein or gelatin hydrolysate as a source of amino acids. We employed a synthetic medium as described by Gladstone,[2] to which the individual amino acids were added. This precaution was taken in order to reduce the impurities to a minimum. A coagulase-positive strain of *S. aureus* isolated from a patient with osteomyelitis was used throughout. Two preparations of cozymase were investigated. One was supplied to us through the courtesy of Dr. D. F. Robertson of Merck and Company, and a second was prepared by one of us (O.M.) according to the method of Jandorf.[6] Professor C. A. Elvehjem of the University of Wisconsin found the activity of the second product to be 81% of the von Euler standard. Qualitative analysis showed that both preparations contained thiamin. The cozymase prepared by us was quantitated by the standard thiacrome method and found to contain 0.16 mg of thiamin chloride per mg of cozymase. As shown later, this amount of thiamin chloride was sufficient to support maximum growth of staphylococci when an optimum amount of nicotinic acid was present.

The basal medium contained all the essential ingredients except thiamin chloride and nicotinic acid. The culture for inoculation was first grown in the complete medium for 24 hours, then centrifuged and the bacterial cells washed with sterile saline before seeding the various tubes with a known number of organisms. The final volume in all the tubes was 10 cc. The degree of bacterial growth after incubation at 37°C was measured according to the turbidity of the contents in each tube. For this purpose, the Evelyn photoelectric

[5] Strauss, E., Dingle, J. H., and Finland, M., *J. Immunol.*, 1941, **42**, 331.

[6] Jandorf, B. J., *J. Biol. Chem.*, 1941, **138**, 305.

colorimeter was used as described previously.[3] Growth was expressed in terms of percentage of light transmitted through a standardized absorption tube. The uninoculated medium was usually water-clear and was utilized as the control standard.

Results. The first group of studies in which an experiment was designed to show the comparative growth stimulus of cozymase, and thiamin chloride and/or nicotinic acid and nicotinamide, in a basal medium containing small and large inoculae is summarized in Table I. A duplicate series of tubes was set up and the results recorded at the end of 24 and 72 hours of growth. From these and other similar observations, the following may be set forth. Thiamin chloride alone provokes only a slight growth response. The addition of only nicotinic acid to the basal medium results in a definite growth, particularly in the presence of a large inoculum. Nicotinamide will serve as a growth stimulus in a smaller concentration than will nicotinic acid, but the same degree of growth is attained when either compound is present in an optimum amount. Maximum, and essentially equivalent growth, was obtained when cozymase was added to the basal medium or when a combination of thiamin chloride and nicotinic acid or nicotinamide was added. A comparison of tubes 3, 4, 5, and 6 would indicate that thiamin chloride is at least one of the added factors responsible for maximum growth resulting with cozymase. A comparison of tubes 5 and 6 containing the small inoculum shows that preformed cozymase will initiate growth more rapidly than a combination of thiamin chloride and nicotinic acid, but that the degree of growth at the end of 72 hours is essentially the

TABLE I.

Comparative Effects of Thiamin Chloride and Nicotinic Acid (or Nicotinamide), and Cozymase on Growth of Large and Small Inoculum of *S. aureus.*

		A* Growth in % of light transmitted		B† Growth in % of light transmitted	
		24 hr	72 hr	24 hr	72 hr
1.	Basal medium	93	94	97	100
2.	" " + 0.0034 μg/cc thiamin chloride	94	88	97	93
3.	" " + 1.23 μg/cc nicotinic acid	75	79	96	88
4.	" " + 5.84 " " cozymase‡	39	41	52	41
5.	" " + 0.0034 μg/cc thiamin chl. + 1.23 μg/cc nicotinic acid	33	41	77	35
6.	" " + 0.00034 μg/cc thiamin chl. + 1.23 μg/cc nicotinic acid	39	43	80	42

*A = Inoculum of 300,000 organisms per cc.
†B = Inoculum of 300 organisms per cc.
‡5.84 μg of cozymase contained 0.0012 μg of thiamin chloride.

same in the two tubes. The lag in initial growth with thiamin chloride and nicotinic acid is very likely due to the fact that nicotinic acid must first be converted into cozymase by the bacterial cells before the growth requirements for bacterial reproduction can be fulfilled.

The second group of studies concerned the comparative effect of thiamin chloride and nicotinic acid, and cozymase upon the bacteriostatic action of sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine in the presence of a large and small inoculum of organisms. The results with a large inoculum (500,000 organisms per cc) are shown in Table II. Under these conditions, bacterial growth was practically the same in the tubes containing cozymase, and thiamin chloride and nicotinic acid. A slight inhibitory effect was produced by sulfathiazole.

The results with a small inoculum of organisms (3500 organisms per cc) are presented in Table III. When the tubes were examined at the end of 43 hours of incubation, the bacteriostatic effect of sulfanilamide was inhibited in the tube containing cozymase, but not

TABLE II.

Comparative Effect of Thiamin Chloride, Nicotinic Acid and Cozymase upon Bacteriostatic Action of Sulfonamide Compounds.
(Inoculum approximately 500,000 organisms per cc.)

	Thiamin Chloride, μg/cc	Nicotinic Acid, μg/cc	Cozymase,† μg/cc	Growth in % of light transmitted at 20 hr
Basal Medium	—	—	—	68*
	—	1.23	—	47
	.034	1.23	—	31
	—	—	9.33	32
Basal Medium + Sulfanilamide 50 μg/cc	—	—	—	70
	—	1.23	—	55
	.034	1.23	—	38
	—	—	9.33	32
Basal Medium + Sulfapyridine 50 μg/cc	—	—	—	70
	—	1.23	—	46
	.034	1.23	—	31
	—	—	9.33	36
Basal Medium + Sulfathiazole 50 μg/cc	—	—	—	68
	—	1.23	—	54
	.034	1.23	—	45
	—	—	9.33	40
Basal Medium + Sulfadiazine 50 μg/cc	—	—	—	69
	—	1.23	—	50
	.034	1.23	—	34
	—	—	9.33	38

*Medium initially slightly cloudy but sterile.
†9.33 μg cozymase contain 0.0019 μg thiamin.

in the tube containing thiamin chloride and nicotinic acid. When duplicate tubes with sulfanilamide were examined at the end of 87 hours, the degree of growth was the same in the tube containing cozymase as in the tube containing thiamin chloride and nicotinic acid. No inhibition of the bacteriostatic effect of sulfapyridine, sulfathiazole or sulfadiazine was exhibited.

A similar experiment was carried out in which the original inoculum was only 60 organisms per cc. When the tubes were examined at the end of 140 hours of incubation, sulfanilamide and sulfapyridine had failed to inhibit the growth of organisms in the tubes containing cozymase, but a complete bacteriostatic effect was exhibited in the tubes with thiamin chloride and nicotinic acid. This difference was not apparent at 72 hours, both sets of tubes showing complete inhibition at this time.

Comment. The ability of the cozymase preparations used in these experiments to replace both thiamin and nicotinic acid is probably due to a contamination of the cozymase with thiamin.

TABLE III.

Comparative Effect of Thiamin Chloride, Nicotinic Acid and Cozymase upon Bacteriostatic Action of Sulfonamide Compounds.
(Inoculum approximately 3500 organisms per cc.)

	Thiamin Chloride, μg/cc	Nicotinic Acid μg/cc	Cozymase, μg/cc	Growth in % of light transmitted at 43 and 87 hrs	
				43 hr	87 hr
Basal Medium	—	—	—	99	100
"	—	1.23	—	83	89
"	.0034	1.23	—	49	50
"	—	—	5.84	47	50
Basal Medium +	—	—	—	100	98
Sulfanilamide	—	1.23	—	96	100
50 μg/cc	.0034	1.23	—	95	43
	—	—	5.84	52	49
Basal Medium +	—	—	—	96	98
Sulfapyridine	—	1.23	—	97	98
50 μg/cc	.0034	1.23	—	97	96
	—	—	5.84	92	87
Basal Medium +	—	—	—	100	97
Sulfathiazole	—	1.23	—	98	96
50 μg/cc	.0034	1.23	—	100	100
	—	—	5.84	96	98
Basal Medium +	—	—	—	98	96
Sulfadiazine	—	1.23	—	97	98
50 μg/cc	.0034	1.23	—	96	98
	—	—	5.84	97	93

5.84 μg cozymase contain .0012 μg thiamin.

A small number of organisms in the presence of thiamin chloride, nicotinic acid and sulfanilamide or sulfapyridine show a lag period in growth. On the other hand, when cozymase is present, growth becomes more readily established. We have assumed that cozymase must be synthesized by the cells before reproduction takes place. Through a mechanism not clearly understood either sulfanilamide or sulfapyridine will interfere with this synthesis. Sulfathiazole and sulfadiazine in the concentration used in these experiments showed complete bacteriostasis for a small number of organisms even in the presence of cozymase. These results confirm the observations of West and Coburn.

When a large number of organisms are used, they are apparently able to synthesize cozymase so rapidly that there is no difference in the degree of growth obtained whether nicotinic acid or cozymase is present. The possibility still remains that the lag period produced by the sulfonamides may be shorter when cozymase is present than when nicotinic acid supplies the growth stimulus. This should be investigated. The bacteriostatic activity of sulfathiazole and sulfadiazine is not completely inhibited by either cozymase or nicotinic acid.

It has been demonstrated by many investigators that the bacteriostatic effect of the sulfonamide compounds is more pronounced when the optimum requirements for bacterial growth are lacking and the number of organisms is small. The foregoing observations are in accord with this concept.

Summary. 1. Two samples of cozymase were tested, and each could replace both thiamin chloride and nicotinic acid, essential growth requirements for the staphylococcus; however, these preparations contained small quantities of thiamin. It has been shown that an adequate amount of thiamin was contained in the cozymase, which explains why maximum growth of staphylococci was obtained in the presence of cozymase, but in the absence of nicotinic acid. 2. When certain experimental conditions are fulfilled, cozymase, but not thiamin chloride and nicotinic acid, will inhibit the bacteriostatic action of sulfanilamide and sulfapyridine against staphylococci.

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Effect of Sulfonamide Compounds upon Staphylocoagulase.*

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Coagulase is a substance elaborated by staphylococci, particularly by the invasive strains. It possesses the property of producing *in vitro* a coagulum in human, citrated plasma. The usual method of testing for the presence of coagulase is to add a loopful of organisms from a 24-hour culture on an agar slant to 0.5 cc of citrated human plasma, and then to incubate the plasma at 37° C. The presence of a coagulum can usually be detected at the end of 3 hours. In many instances, a solid clot will be formed.

While investigating the antistaphylococcal activity of sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine, it was observed on a number of occasions that the usual effect of coagulase was reduced or absent in the presence of high concentrations of these compounds. It was assumed that the compounds interfered with the metabolism of the bacterial cells, and coagulase was not being formed as readily. However, there remained the possibility that the drugs may have acted directly upon coagulase. Since there is some evidence that the sulfonamide compounds exert their antibacterial effect by interfering with the enzyme activity of the bacterial cells, it seemed important to determine what effect the drugs had upon staphylocoagulase when the substance was separated from the cells. While the true nature of staphylocoagulase is not known, Cruickshank states it has some of the characteristics of an enzyme.[1] It is known that active staphylocoagulase may be present in sterile, broth filtrates of staphylococci.[2, 3]

Methods and Materials. Normal human plasma containing sodium citrate in a concentration of 0.3% was obtained from the blood bank of the University of Minnesota Hospitals. Three coagulase-positive strains of *S. aureus* isolated from patients with severe infections were used. In preparing a bacteria-free, broth filtrate containing staphylocoagulase, various methods were investigated and

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1 Cruickshank, R., *J. Path. and Bact.*, 1937, **45**, 294.

2 Walston, H. D., *J. Hygiene*, 1935, **35**, 549.

3 Fisher, A. M., *Bull. Johns Hopk. Hosp.*, 1936, **59**, 393.

the following method adopted. A loopful of each culture was seeded in 100 cc of veal infusion broth contained in sterile, pyrex flasks. The 3 cultures were incubated at 37°C for 7 days. At the end of this time, they were centrifuged, and the supernatant fluid sterilized with a Berkefeld N filter. Approximately 100 cc of physiologic sodium chloride solution was first permitted to run through the candle in order to remove any traces of calcium that might be present. The filtrate was then tested for sterility and stored in the refrigerator. Preliminary observations with different broth filtrates and samples of plasma were routinely made in order to determine the presence of coagulase activity. It was found that the coagulating property of coagulase in the filtrates varied considerably with different samples of plasma. In many instances, after the broth filtrate was incubated with plasma for several hours, no coagulum could be detected. Throughout the present study, a total of 52 different samples of plasma were used.

Because we had previously observed that sulfathiazole and sulfadiazine inhibited the growth of staphylococci to a greater degree than either sulfapyridine or sulfanilamide, the first mentioned drugs were first selected for study.[4] The sodium salts of sulfathiazole and sulfadiazine were used because of their greater solubility. Comparative observations were also made with solutions of sulfathiazole and sulfadiazine. When the higher concentrations of the compounds were desired in plasma, suspensions of the drugs were utilized. The first group of studies was carried out in the following way: Small glass tubes contained 0.5 cc of citrated plasma, 0.25 cc of the broth filtrate, and either 0.25 cc of physiologic, sterile sodium chloride solution, or 0.25 cc of a solution of a sulfonamide compound. The final dilution in all of the tubes was 1.0 cc. The final concentrations of the sulfonamide compounds in the plasma were 10, 100, 250, 500, and 1000 mg per 100 cc. The tubes were incubated in a water bath at 37°C. They were examined at various time intervals for the presence of a coagulum.

In another series of studies, the comparative effects of sulfanilamide, sulfapyridine, sulfathiazole and sulfadiazine were noted. The drugs were added as a saline suspension, in those instances where high plasma concentrations were desired. Otherwise, the method used was the same as described above.

Results. Table I shows the results obtained with varying concentrations of sodium sulfathiazole and sodium sulfadiazine. These results were obtained consistently with several different samples of

[4] Spink, W. W., and Jermsta, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 395.

TABLE I.
Effect of Varying Concentrations of Sodium Sulfathiazole and Sodium Sulfadiazine upon Action of Staphylocoagulase in Citrated Human Plasma.

Time of Incubation	Control	Na Sulfathiazole mg per 100 cc					Na Sulfadiazine mg per 100 cc				
		1000	500	250	100	10	1000	500	250	100	10
15 min.	0	0	0	0	0	0	0	0	0	0	0
30 "	0	0	0	0	0	0	0	0	0	0	0
45 "	0	+	+	+	+	0	0	0	0	0	0
60 "	0	+	+	+	+	+	0	0	0	0	0
90 "	+	+	+	+	+	+	0	0	0	0	0
2 hr.	+	+	+	+	+	+	0	0	0	+	+
3 "	+	+	+	+	+	+	0	0	0	+	+
6 "	+	+	+	+	+	+	0	0	0	+	+
18 "	+	+	+	+	+	+	0	0	0	+	+

+ = Coagulum.
0 = No coagulum.

plasma and broth filtrates. The outstanding features are that sulfathiazole in concentrations between 100 and 1000 mg per 100 cc accelerated the coagulating activity of staphylocoagulase, while sulfadiazine in the same concentrations inhibited this activity. Control tubes containing only plasma and the sulfonamides did not reveal any clotting effect. No coagulum was present in tubes with a mixture of plasma, veal infusion broth and either sulfathiazole or sulfadiazine. The amounts of the sodium salts of the two compounds which were employed did not result in any significant change in the hydrogen-ion concentration of the plasma.

The comparative effects of crystalline sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine in varying concentrations are shown in one of the experimental results in Table II. Again, it is to be noted that sulfathiazole accelerated the coagulating activity of staphylocoagulase, whereas, sulfadiazine acted in an inhibitory manner. In this particular experiment, it would appear that a concentration of 100 mg per 100 cc of sulfadiazine accelerated coagulation, but this observation is inconsistent with other experiments. Sulfanilamide operated in a manner similar to sulfathiazole, while the effect of sulfapyridine approximated the results in the control tubes and in some instances inhibited the coagulating effect.

A series of observations were made to determine if p-aminobenzoic acid would inhibit the action of the sulfonamides upon staphylocoagulase. Concentrations of p-aminobenzoic acid up to 20 mg per 100 cc did not show any consistent results. When concentrations of 100 mg per 100 cc were used, it was found that all of the compounds had an accelerating effect in the presence of p-aminobenzoic acid. This compound, when studied alone in the presence of a broth

TABLE II.

Effect of Various Concentrations of 4 Sulfonamide Compounds upon Action of Staphylocoagulase in Citrated Human Plasma.

Hrs of Incubation	Control		Sulfanilamide mg per 100 cc				Sulfapyridine mg per 100 cc				Sulfathiazole mg per 100 cc				Sulfadiazine mg per 100 cc			
	A	B	1000	500	250	100	1000	500	250	100	1000	500	250	100	1000	500	250	100
1	0	0	2+	0	0	0	0	0	0	0	3+	3+	3+	0	0	0	0	0
1½	0	0	3+	2+	2+	1+	0	0	0	0	3+	3+	3+	1+	0	0	0	3+
2	0	0	3+	2+	3+	1+	0	0	0	0	3+	4+	4+	1+	0	0	0	3+
2½	0	0	4+	4+	4+	2+	0	0	0	1+	3+	4+	4+	1+	0	0	0	3+
3	1+	0	4+	4+	4+	3+	2+	1+	1+	1+	3+	4+	4+	1+	0	0	0	3+
3½	1+	1+	4+	4+	4+	3+	2+	1+	1+	1+	3+	4+	4+	1+	0	0	0	3+
4	1+	1+	4+	4+	4+	3+	2+	1+	2+	1+	3+	4+	4+	1+	0	0	0	3+
5	1+	1+	4+	4+	4+	3+	3+	3+	3+	3+	3+	4+	4+	1+	0	0	0	1+
6½	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	4+	4+	2+	3+	0	3+	4+
24	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	2+	4+	1+	4+	4+

1+ to 4+ = Minimum and maximum clotting of plasma.

filtrate and plasma, had a markedly accelerating coagulating effect, similar to sulfanilamide.

No explanation is put forward to account for the influence which the sulfonamides have upon staphylocoagulase activity. It should be emphasized that the presence of significant traces of calcium in the sulfonamide solution was ruled out.

Summary. 1. Sulfathiazole and, to a slightly lesser extent, sulfanilamide accelerated the clotting action in citrated plasma of staphylocoagulase contained in a sterile broth filtrate. Sulfadiazine markedly inhibited the coagulating effect, while sulfapyridine had only slight inhibitory activity. 2. Para-aminobenzoic acid operated like sulfanilamide and accelerated the coagulating action. 3. Broth filtrates with staphylocoagulase did not cause coagulation of every sample of normal, citrated human plasma. The degree of coagulation also varied.

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Influence of Tissue on Bacteriostatic Effect of Paranitrobenzoic Acid.*

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King and Henschel[1] reported that paranitrobenzoic acid exerted a bacteriostatic action against Type II pneumococcus in tissue culture clots which compared favorably with that of sulfathiazol in the same medium. Earlier the same authors[2] reported that " . . . . the characteristic effects of sulfanilamide on beta hemolytic streptococci are markedly inhibited by the products of tissue-breakdown."

In this paper we report the results of a study on the inhibition of the bacteriostatic effect of the sodium salt of paranitrobenzoic acid by the products of tissue-breakdown. The details of the tissue culture technic used have been described in the above papers.

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1 King, J. T., and Henschel, A. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 400.

2 *Ibid.*, 1939, **41**, 208.

Pneumococci, Type II, were cultured in rabbit serum extract of 8-day chick embryos; incubation 3-5 hours at 37.5° C. The culture was then rapidly diluted through Tyrode solution into the serum extract to make a dilution of 10^{-8}.

The suspension was well mixed and divided into two equal portions to one of which sufficient sodium paranitrobenzoate was added to make 15 mg % in the final culture; to the other portion an equal volume of saline was added. The drug and saline were sterilized by Berkefeld filtration. pH of both drug solution and saline was adjusted to 7.6 before using.

To determine the degree of bacteriostasis 6 cultures were planted from the suspension without drug and 6 from the suspension containing the drug. Each culture consisted of 3 drops of suspension and one drop of heparinized rabbit plasma.

To determine the degree of bacteriostasis in the presence of disintegrating tissue, a similar set was then planted, each culture containing a sterile fragment of chick heart from a 17-19-day embryo. The fragments which were matched for size and shape measured 1.5-2.0 mm in diameter.

Cultures were incubated as lying-drop preparations for approximately 15 hours at 37.5° C in a special incubator previously described.[3] Measurement of the diameter of the colonies was made at 60 × (114 eyepiece units = 1 mm).

The results are given in Table I.

It may be noted that addition of even a small tissue fragment to the drug-free control medium accelerates the growth rate. The difference is large when few colonies are present and may be rather slight when the colony count is high.

In this concentration the drug exercised a consistent inhibitory effect both in the presence and absence of tissue but in all experiments the effectiveness of the drug was markedly reduced by a small tissue fragment.

TABLE I.

Colonies per cc	Cell free		With tissue		Inhibition %	
	Control	PNO_2	Control	PNO_2	Cell free	With tissue
424	56.8*	21.7	66.7	57.3	61.8	14.1
592	79.4	23.8	94.4	69.8	70.0	26.0
2812	38.0	18.8	43.9	32.7	50.5	25.4
45	117.7	41.6	213.5	140.8	64.7	34.1
454	54.7	30.5	74.2	57.8	44.3	22.1
696	48.0	25.8	70.7	56.7	46.2	19.8

*Eyepiece units.

[3] King, J. T., *Arch. f. Exp. Zellforsch.*, 1937, **20**, 208.

The mechanism of action of the sulfa compounds and probable explanations for their inhibition by various factors are thoroughly discussed in recent papers by Long,[4] by Kohn and Harris,[5] and by Harris and Kohn.[6] Presumably the same reasoning may be applied to paranitrobenzoic acid.

Conclusion. The bacteriostatic effect of paranitrobenzoate on the pneumococcus is markedly reduced by the products of disintegrating tissue.

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Penetration of Sulfonamides Through Intact Skin by Iontophoresis and other Means of Local Application.

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This report presents some preliminary studies in which penetration by topical application of various sulfonamides has been measured by tissue analysis. It was thought that iontophoresis (electrolytic ion transport) might be an effective means of forcing the anion of sulfonamide salts into the skin. Penetration by iontophoresis was, therefore, compared with that from wet dressings and from an ointment, in the rat, rabbit, and human. The effect of circulation and the depth of penetration were estimated.

Methods. The experimental animals were anesthetized with nembutal and the hair removed with clippers. In the iontophoresis experiments, Canton flannel (6 oz grade) was wetted with the test solution and applied to the skin. Metal gauze electrodes were firmly applied over the flannel as the negative pole. The positive electrode was applied to one of the extremities, and currents of 1-2 milliamperes at 110-125 volts D.C. were applied.

Penetration from wet dressings (gauze or flannel) without current passage, was similarly studied in comparable areas on the same animal or on different animals.

[4] Long, P. H., *Sigma Xi Quarterly*, 1941, **29**, 149.

[5] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1941, **73**, 343.

[6] Harris, J. S., and Kohn, H. I., *J. Pharm. and Exp. Therap.*, 1941, **73**, 383.

Penetration from an ointment base was also compared. 5% sodium sulfathiazole (NaSAT) in "Aquaphor"* was used in these studies. 5% NaSAT and saturated aqueous (1.8%) calcium sulfathiazole (CaSAT)† were used in the iontophoresis and wet dressing experiments.

At the termination of the application period, biopsies or autopsies were performed after exhaustively washing the skin with distilled water and alcohol. Tissue aliquots were removed from within the areas of applications, washed several times through water and alcohol, blotted, weighed, thoroughly macerated with sand in a mortar, diluted to appropriate volumes to contain 3% trichloracetic acid, and filtered. The filtrates were passed back through the filters 5 times, since the fourth filtrates were found to contain no measurable sulfathiazole (SAT). Recoveries of known amounts of SAT added to tissue were obtained within 1-2%. The animal tissue aliquots weighed 0.5-2.0 g and the human biopsies, 0.2-0.6 g. The latter were performed under novocaine anesthesia by a punch, the wounds healing by secondary intention. Free SAT was colorimetrically determined in the filtrates by the method of Bratton and Marshall.[1]

Results. 1. *Penetration of CaSAT and NaSAT into the intact skin of the rat by iontophoresis and from ointment.* Penetration of a saturated solution of CaSAT (1.8%) from negative electrodes was compared with that of 5% NaSAT ointment applied more distally on the same animal. 30 x 25 mm electrodes were used in iontophoresis. An equal area was covered with the ointment. The rats were sacrificed at various times and skin aliquots were analyzed. The results appear in Table I A. Although comparison is made of two different sulfonamide salts at different concentrations and differently applied, the tissue concentrations found were apparently identical. The higher value for iontophoresis versus ointment after 2 hours may not be significant since too few determinations were made and since individual variations in duplicate determinations are large, as seen in Tables II and III.

2. *Penetration of NaSAT into the intact skin of rats by iontophoresis and from wet dressings and ointment.* These experiments were performed as above, but for longer periods, and using the same

* Duke Laboratories, Inc. Consists of 5% cholesterol in petrolatum. 5 g of NaSAT were suspended in 5 ml of water and worked into the "Aquaphor" with a spatula.

† Kindly supplied by Doctor W. W. Spink, of the Department of Internal Medicine, this institution.

[1] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE I.
Penetration of CaSAT and NaSAT into Intact Skin of Rats.
A. Penetration of CaSAT by Iontophoresis compared with that of NaSAT from Ointment.

Treatment	Time applied (hr) No. of rats	Tissue SAT mg% 0.5	1.0	1.5	1.7	2.0
Iontophoresis (Sat'd CaSAT)	5	6	9	11	29	58
Ointment (5% NaSAT)	6	7	9	10	14	27
					40	
					Av. 27	

B. Penetration of NaSAT by Iontophoresis and from Wet Dressings and Ointment.

Treatment	Time applied (hr) No. of rats	Tissue SAT mg% 2.0	4.0	6.0
Iontophoresis (5% NaSAT)	6	53	108	69
		68	55	77
		Av. 60	Av. 81	Av. 73
Wet dressing (5% NaSAT)	6	62	106	158
		132	91	110
		Av. 97	Av. 103	Av. 134
Ointment (5% NaSAT)	1	27	—	—

concentration of the same sulfonamide salt (NaSAT) throughout. Duplicate determinations were made, using 2 animals for each type and time period of application. Tissue aliquots were analyzed after 2, 4, and 6 hours. One ointment determination from Table I A is included. The results are seen in Table I B. Although the individual variations were large, it is apparent that the tissue concentration tended to plateau after 2-3 hours (see also Table I A), at an average concentration of ca 50 times less than that of the applied solutions. No large differences in penetration of SAT salts by iontophoresis and from wet dressings are apparent until 6 hours, when the latter shows better penetration. At 2 hours, penetration from ointment is inferior to that from wet dressings or iontophoresis.

3. *Depth of penetration of CaSAT through skin of rabbit by iontophoresis and from wet dressings.* Areas 6 x 8 cm on the thighs of a rabbit were treated with 5% NaSAT by iontophoresis and by wet dressing for 3 hours. Aliquots of skin, subcutaneous muscle, the deeper muscles, and blood, were analyzed for SAT. The results appear in Table II A. A 40% greater concentration of SAT was obtained in the skin by iontophoresis than by wet dressing, which

TABLE II.

A. Depth of Penetration of CaSAT through Skin of Rabbits by Iontophoresis and from Wet Dressings.
3 hrs application. 6-lb rabbit. Thigh. (Detail in text.)

Treatment	Tissue SAT mg%			
	Skin	Subcut. muscle	Deep muscle (biceps, adductor)	Blood
Iontophoresis (Sat'd CaSAT)	312	64.5	trace	trace
Wet dressing (Sat'd CaSAT)	224	0.7	"	"

B. Penetration of CaSAT and NaSAT into Intact and Ligated Legs of Rats by Iontophoresis and from Ointment.
1.7 hrs application. 2 rats. Thigh. (Detail in text.)

Rat No.	Treatment	Tissue SAT mg%		
		Leg	Skin	Deep muscle (biceps)
1.	Iontophoresis (Sat'd CaSAT)	Left (ligated)....24		trace
		Right (intact)....29		"
2.	Ointment (5% NaSAT in "Aquaphor")	Left (ligated)....19		"
		Right (intact)....40		"

we consider a small difference (compare Fig. 1). The iontophoresis seemed to produce a higher concentration in the subcutaneous muscle in the rabbit. Immeasurably small quantities of SAT were found in the deeper muscles and in the blood. Very large concentrations of SAT may be obtained in the intact skin by topical wet application, while the blood level is negligibly small.

4. *Penetration of CaSAT and NaSAT into the intact and ligated legs of rats by iontophoresis and from ointment.* The left legs of 2 rats were tightly ligated and the lateral surface of the left thigh treated with 5% NaSAT ointment in 1 rat, and with saturated aqueous CaSAT in the other. The right thighs acted as controls with intact circulation. Table II B shows the results. Ligation has no effect on the penetration of NaSAT by iontophoresis for 100 minutes, whereas ligation may have caused some impairment in penetration of CaSAT. Again we do not feel that this difference is significant, due to paucity of data and the individual variations seen in the tables. We feel that in general, in the rat, ligation has no effect on penetration (see discussion at end of paragraph 6). The deeper muscle layers contained negligible amounts of SAT.

5. *Penetration of NaSAT into human skin by iontophoresis, wet dressings, and ointment.* 7x7 cm areas were treated for 3 hours with 5% NaSAT by the 3 methods, and duplicate punch biopsies from each area analyzed for SAT. Table II shows the results. No difference in penetration of NaSAT was observed by ionto-

TABLE III.
Penetration of NaSAT into Intact Human Skin.
Normal skin from thigh. Duplicate punch biopsies through the corium. Biopsy weights 30-50 mg. 5% NaSAT applied for 3 hours.

	Tissue SAT mg%		
	Iontophoresis	Wet dressing	Ointment
Case 1 (64 yr female, normal weight)	55	57	trace
	—	45	"
Case 2 (26 yr male, corpulent)	36	33	"
	60	87	"
	—	—	—
	av. 50	av. 55	av. "

phoresis compared with that from wet dressing, and penetration from ointment was negligible. In this latter respect human skin is less permeable than rat skin, although penetration of NaSAT in rat skin from ointment was also less than from wet dressings or iontophoresis.

6. *Penetration of CaSAT and NaSAT into the skin of live and dead rabbits by iontophoresis and from wet dressings.* Penetration of saturated aqueous CaSAT (1.8%) from wet dressings and by iontophoresis, into the skin of the back was studied for 1 and 3 hours in one rabbit; and that of 5% NaSAT by iontophoresis and from wet dressings in one anesthetized and in one dead rabbit. Biopsies were performed after 1, 2, and 3 hours. Fig. 1 depicts the

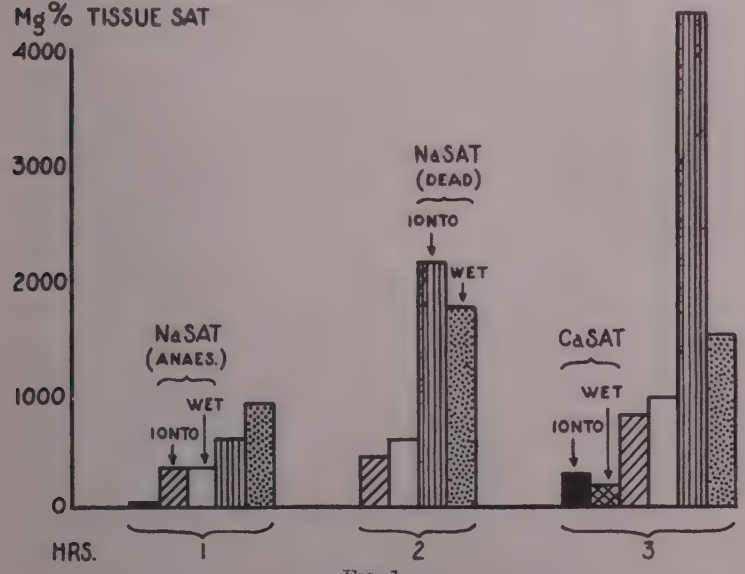

FIG. 1.

results. The skin concentration of SAT after iontophoresis and wet dressing applications of the CaSAT were not noticeably different after 3 hours, and were less than those obtained by similar applications of 5% NaSAT. Slightly higher levels were obtained after 2 and 3 hours' wet dressing application than by iontophoresis in the anesthetized animal, but iontophoresis effected much higher concentrations than wet dressings in the skin of the dead animal after 3 hours, reaching a concentration of 4%. This observation may explain the lack of differences between iontophoresis and wet dressing applications seen in Tables I-III. Electroösmosis of water toward the negative pole may counteract electrical ion transport of sulfonamide anions in the live animal with normal skin permeability and circulation, and in the edematous ligated limbs of rats; where it does not in the dead animal. More experiments than are reported in this preliminary paper will be necessary to firmly establish this point.

A second observation of importance is that within the periods of time studied, NaSAT attains markedly higher concentrations in rabbit skin than in human or rat skin.

Summary. 1. The penetration of sodium sulfathiazole (NaSAT) into the intact skin of rats, rabbits, and humans, up to 6 hours' application by iontophoresis and from wet dressings, is essentially equal, while that from an ointment base is less than either of these. Penetration is somewhat better from wet dressings than by iontophoresis after longer times.

2. Saturated (1.8%) calcium sulfathiazole (CaSAT) penetrates the intact skin of rats to the same extent by iontophoresis as from 5% NaSAT ointment.

3. Iontophoresis of NaSAT through the intact skin of rabbits produces a greater concentration of SAT in subcutaneous muscle than wet dressing application, but the concentrations in deep muscle and blood are negligibly small.

4. Impaired circulation in rats by ligation of the limbs, has little effect on penetration of CaSAT and NaSAT into these limbs by iontophoresis and from ointment.

5. Penetration of CaSAT and NaSAT by iontophoresis and from wet dressings into rabbit skin is essentially the same. Penetration of NaSAT into the skin of dead rabbits by iontophoresis is greater than from wet dressings.

13695

Distribution of Chlorides and Bicarbonates in Blood Following Addition of Hydrochloric Acid.

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A recent study of the distribution of chlorides between the plasma and red cells in cases of arterial hypertension (Apperly and Cary[1]) led us to question some of the usually accepted theories concerning electrolyte distribution in blood generally. Van Slyke and others,[2] as the result of experiments and considerations of thermo-dynamics, have formulated certain laws regarding the distribution of water and ions between red cells and plasma under various conditions. One of these laws can be expressed thus:

$$\frac{(Cl)_c}{(Cl)_s} = \frac{(HCO_3)_c}{(HCO_3)_s} = \frac{(H)_s}{(H)_c} = \frac{(B.Hb)_c}{2 \cdot (A)_s}$$

where $(Cl)_c$, $(HCO_3)_c$, and $(Cl)_s$, $(HCO_3)_s$, etc., represent concentrations in millimols per liter of water in cells and in plasma respectively, (B.Hb) refers to concentration of base bound by hemoglobin and (A) to concentration of total monovalent ions (largely Cl and HCO_3). From the above it follows that these ratios rise or fall with increasing or decreasing H-ion concentration until finally the ratios equal unity at pH 6.6, the isoelectric point of hemoglobin.

Although these laws were worked out on horse's blood *in vitro*, they have since been confirmed, in the main, on the blood of man and other animals *in vitro*, after the use of varying pressures of CO_2 to produce changes in pH, and in dogs *in vivo*, following the intravenous injection of hydrochloric acid (Harkins and Hastings[3]). We have also found the $(Cl)_c : (Cl)_s$ and $(HCO_3)_c : (HCO_3)_s$ ratios to vary in a roughly parallel manner following a ketogenic diet for 18 days (Apperly and Norris[4]) and as the result of elevation to the moderate altitude of 6000 feet (Apperly[5]). In severe disease the laws also hold good (Muntwyler, *et al.*[6]).

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1 Apperly, F. L., and Cary, M. K., *Am. J. Med. Sci.*, 1937, **194**, 352.

2 Van Slyke, D. D., *Factors Affecting the Distribution of Electrolytes, Water and Gases in the Animal Body*, Lippincott Co., Philadelphia and London, 1926.

3 Harkins, H. N., and Hastings, A. B., *J. Biol. Chem.*, 1931, **90**, 565.

4 Apperly, F. L., and Norris, J. H., *Am. J. Med. Sci.*, 1933, **185**, 802.

5 Apperly, F. L., *Am. J. Physiol.*, 1938, **122**, 179.

6 Muntwyler, E., Myers, V. C., and Way, C. T., *J. Biol. Chem.*, 1931, **92**, 721.

The laws, however, are only approximations. Thus Van Slyke, *et al.*,[7] found by direct observation that:

$$\frac{(H)_s}{(H)_c} = 0.77\frac{(Cl)_c}{(Cl)_s} = 0.62\frac{(HCO_3)_c}{(HCO_3)_s}$$

Further, the ratios do not reach unity at the pH corresponding to the isoelectric point of hemoglobin. Various explanations, such as an incomplete dissociation of Cl, or the formation of carb-hemoglobin in the cells, with an apparent rise in the $(HCO_3)_c$: $(HCO_3)_s$ ratio, have been invoked to explain these discrepancies.

Methods. In an endeavor to avoid some of these possible sources of error we have introduced certain modifications.

(a) In all previous work the blood changes in pH, and consequently in redistribution of ions, have been brought about by the addition of H_2CO_3, HCl or other acid, all involving the addition of 2 different ions, and, generally, an altered total ionic concentration in the blood, so that the variations in the curves obtained were each the resultant of several factors. This has been avoided by comparing bloods in which only the kation was changed, *i. e.*, the Na ion was gradually replaced by H ions, all other ions remaining constant.

(b) The CO_2 combined with reduced hemoglobin as a carbamino compound (carb-hemoglobin) accounts for about 6 volumes % in an average blood sample. In oxygenated blood, however, the carbamino CO_2 is reduced to about one-third of this, and falls to even lower values with fall in pH (Ferguson and Roughton[8]). Since in our experiments all bloods were oxygenated, and in most cases acidified with HCl, the carbamino CO_2 held must therefore, according to these authors, have been very small indeed. The slight, though constant differences between our curves for cell CO_2 and plasma CO_2 correspond closely in magnitude to the carbamino CO_2 content found by these workers and, in further discussions, will be ignored.

Mixtures were made by adding 1.5 cc of 0.5 N chloride solution to 23 cc of oxalated or defibrinated dog's blood. The composition of the chloride solutions varied in the relative amounts of HCl and NaCl, so that the resultant mixtures with blood varied only in their H and Na concentrations. The Cl mixture was slowly added and well mixed. The blood was then transferred to a 400 cc separatory funnel, saturated with the alveolar air of one of us, and the following then determined, *viz.*: Cl (Eisenman's modification of Van Slyke's

[7] Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, **65**, 701.

[8] Ferguson, J. K. W., and Roughton, F. J. W., *J. Physiol.*, 1934, **83**, 87.

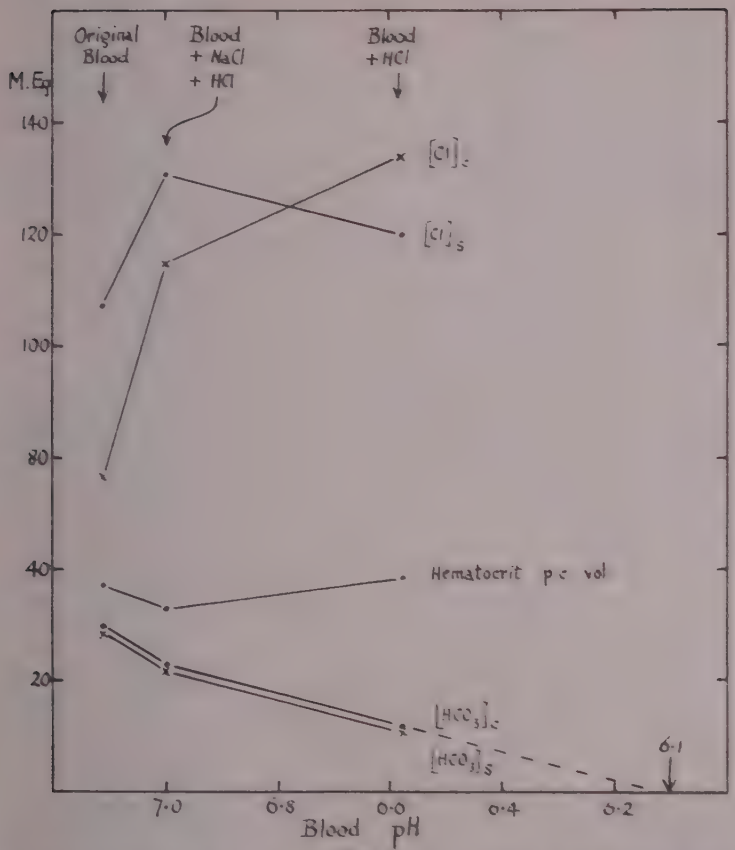

FIG. 1.

Showing alterations in Cl and HCO_3 concentrations in both plasma and red cells of blood following the addition of equal volumes of equi-molecular mixtures of NaCl and HCl.

method[9]) and CO_2 contents (Van Slyke and Cullen[10]) of plasma and whole blood: hematocrit volume of red cells: water content of plasma and red cells (by dry weight): and pH (Coleman glass electrode) of the various mixtures. From these figures the Cl and HCO_3 were calculated as mM per liter of plasma water and of red cell water. Where applicable, all results were corrected as for 38°C.

After many preliminary trials, 10 complete experiments were carried out. In each, (HCO_3) and (Cl) were plotted against pH since below pH 7.0 at any rate, the curves obtained were nearly

[9] Eisenman, A. J., *J. Biol. Chem.*, 1929, **82**, 411.

[10] Van Slyke, D. D., and Cullen, G. E., Jr., *J. Biol. Chem.*, 1917, **30**, 289.

straight lines (Van Slyke, *et al.*[11]). Fig. 1 is typical of the group.

Results. (a) The CO_2 content. The $(HCO_3)_c$ and $(HCO_3)_s$ curves nearly coincide, *i. e.*, the ratio of the former to the latter equals unity irrespective of pH. When these curves are traced to the right they reach their lowest values at about pH 6.1. If the blood is saturated with alveolar air as described above the CO_2 values are about 2 mM at pH 6.1, but if with atmospheric air this value falls to about 1 mM. These figures were confirmed in other experiments with plasma alone by adding sufficient acid to carry the mixture below pH 6.1. In these and other experiments we find a bend in both the plasma and red cell CO_2 curves somewhere between pH 7.0 and 7.2, below which the curves are almost straight lines.

(b) The Chlorides. With diminishing pH the red cell chloride curve rises and the plasma chloride curve falls, but the most careful work failed to give any constant pH point at which the curves crossed, *i. e.*, where $(Cl)_c - (Cl)_s = 1$. In general, the pH at which the crossing occurred seemed to depend upon and to vary roughly with the red cell content of the blood.

(c) The hematocrit volume shows a small but steady rise with falling pH, due, as is well known, to transfer of water from plasma to red cells.

Discussion. In general these results seem to be confirmed when we plot, in similar manner, some of the results of other workers in which acid other than CO_2 was added to blood *in vitro* or *in vivo* and when sufficient data were available for values below pH 7.0. In Harkins and Hastings' paper[9] experiments 2 and 3 and Fig. 7 all show practical disappearance of CO_2 at about pH 6.1. Again at normal blood pH, the average value given by several other authors for cell water is about 65% and for plasma water about 92%. Since the ratio 65/92 equals about 0.70 the same figure obtained by Apperly and Cary[7] for the value of the ratio $(HCO_3)_c : (HCO_3)_s$ for many bloods (when no allowance was made for cell and plasma proteins) it follows that in normal blood the ratio $(HCO_3)_c : (HCO_3)_s$ calculated as concentrations per unit of cell water, must approximate unity. Further, Peters[12] gives the value of $(HCO_3)_c - (HCO_3)_s$ at normal blood pH as 0.027–0.028 which also, for practical purposes, equals unity. Since both the $(HCO_3)_c$ and $(HCO_3)_s$ curves are practically straight lines, and both reach very small values or zero at about pH 6.1, it follows that the 2 curves approximately coincide, and therefore that the values of the ratio at all pH levels less than

[11] Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1925, **56**, 765.

[12] Peters, J. P., *Body Water*, Baltimore, Tindall and Cox, London, 1935, p. 99.

normal approximately equal unity when the acid added is one other than CO_2. The HCO_3 ion would therefore appear to be freely diffusible into and out of the cell wall. The differing values of the ratio obtained by other observers, using different pressures of CO_2, suggest that the formation of carbhemoglobin has complicated the picture.

We have not yet further investigated the apparent dependence on the hematocrit value (or red cell content) of the pH at which $(Cl)_c/(Cl)_s = 1$. As already stated, this pH appears to vary directly with the hematocrit value. It would seem reasonable to suggest, however, other conditions being equal, that blood with high red cell content, *i. e.*, better buffered, would take up more CO_2 when saturated with alveolar air, and therefore result in a larger transference of Cl from plasma to red cells. This in turn would mean a more rapid rise in the $(Cl)_c$ curve and a correspondingly rapid fall in the $(Cl)_s$ curve, so that the crossing of the curves is shifted to left. Conversely, the pH at which $(Cl)_c/(Cl)_s = 1$ in anemic blood would be expected to shift to the right, *i. e.*, at a lower pH.

Summary. An investigation has been made of certain aspects of Van Slyke's laws concerning the distribution of ions between red cells and plasma at different pH values. When hydrochloric acid was used to bring about variations in blood pH, losses of CO_2 from plasma water and cell water were equal, so that the ratio $(HCO_3)_c/(HCO_3)_s$ remained unchanged and equal to unity at all pH values. The values of $(Cl)_c/(Cl)_s$ at different pH values did not parallel the values of $(HCO_3)_c/(HCO_3)_s$ but gradually rose to unity at values of pH which varied, the actual value appearing to depend upon and to vary with the red cell content of the blood.

13696 P

A New Method of Administering Heparin.*

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In the course of studies on experimental subacute bacterial endo-

* This work was made possible through the coöperation of the Roche-Organon Company of Nutley, N.J., which supplied all the necessary materials. We wish to express our appreciation to Drs. Ralph D. Shaner and Leo Pirk of that organization for their interest and helpful suggestions.

carditis, attempts were made to produce the disease in heparinized rabbits. The methods for heparinizing humans, namely, by continuous venoclysis or by repeated intravenous injections, were virtually impossible of adaptation for the rabbit. However, rabbits will develop and maintain a prolonged coagulation time over a protracted period with repeated subcutaneous injections of heparin, the average daily requirement being about 45 mg of commercial heparin† administered in fractional doses.[1]

In order to eliminate the disturbing abrupt rises, sharp peaks and unpredictable drops in coagulation time as well as the technical difficulties incidental to fractional daily dosage, a simpler means had to be devised for continuous heparinization by the subcutaneous route.

In view of the success obtained with pellet and capsule implantation of hormones,[2] similar experiments were pursued with heparin. Pellets containing 50 mg and glass capsules tightly packed with 100 mg of heparin were implanted under sterile precautions subcutaneously or subfascially through a small skin incision. While rabbits could be heparinized in this fashion, this approach had to be abandoned for the following reasons: The results with glass capsules were erratic. On some occasions a spectacular but shortlived effect was obtained; at other times, the results were negative and when the capsule was removed subsequently it was discovered that practically none of the heparin had been acted upon.

Additional shortcomings were: The necessity for instrumental procedure; the rapid absorption of pellets with resultant inordinately delayed coagulation time and subsequent precipitate drop; the presence at times of local hemorrhage and the obvious impracticability of sustaining heparin effects over prolonged periods.

To accomplish a slower and more equable absorption of heparin, the Pitkin menstruum, composed of gelatin, glacial acetic acid, glucose, and water in definite proportions was suggested as a vehicle. Ampuls containing varying amounts of heparin and vasoconstrictor elements in the Pitkin menstruum were prepared. All the ingredients apart from heparin were found to be inactive in control experiments.

The contents of the ampul are liquefied at 55° C. drawn up through a 1½ inch, 18 gage needle into a previously warmed sterile 2 cc syringe and immediately injected subcutaneously. The material congeals promptly following inoculation.

† The heparin used is marketed by the Roche-Organon Company under the trade name of "Liquaemin."

[1] Loewe, L., Rosenblatt, P., and Lederer, M., to be published.

[2] Deanesley, R., and Parker, A. S., *Proc. Roy. Soc., Ser. B.*, 1937, 124-279.

TABLE I.
Heparin—Pitkin Formulæ.

	L.P.	L.P.-1	L.P.-2	L.P.-3	L.P.-4
Cryst. sodium salt of heparin	100.0 mg	50.0 mg	50.0 mg	25.0 mg	25.0 mg
Epinephrine hydrochloride	1.0	0.25	0.1	0.25	0.1
Ephedrine sulfate	25.0	10.0	5.0	10.0	5.0
Chlorbutanol	0.5	0.5	0.5	0.5	0.5
Eucupin dihydrochloride	1.0	1.0	1.0	1.0	1.0
Pitkin menstruum q.s. ad.	1.0 cc	1.0 cc	1.0 cc	1.0 cc	1.0 cc

The initial formula (Table I—L.P.) contained 100 mg of heparin. The excessive content of epinephrine and ephedrine, however, induced in all of the 8 animals a severe vasoconstrictor effect. This was overcome by adjustment of the constrictor elements. The dose of heparin was also varied in an effort to reduce the total amount of the drug required to obtain the desired result (Table I—L.P.-1, 2, 3, 4).

The individual effects of the various formulae were evaluated in the course of 62 experiments performed on 52 rabbits. As compared with the average normal of 9 minutes for rabbits‡ a coagulation time of 30 minutes to 2 hours was considered a satisfactory heparin response. In practically every instance an acceptable prolongation of coagulation time was obtained. The effects of a single ampul lasted from 24 to as much as 72 hours.

In several animals heparinization was initiated with 50 mg of heparin (Table I—L.P.-1 or 2) and continued as required with 25 mg doses (Table I—L.P.-3 or 4). In this manner it was possible to maintain adequate heparinization over a two-week period with as little as 100 mg of heparin. This contrasts with a 2-week requirement of 630 mg commercial heparin given subcutaneously in fractional daily doses of about 45 mg.

Further revamping of the formula may yield more prolonged effects. Experience with humans may disclose the need for additional alteration in the proportions of the various ingredients. Such experiments in humans are being pursued with gratifying results.

The implications and applications of this approach are self-evident. A retarding influence to the more widespread use of heparin is the present cumbersome technic of intravenous administration. This has been circumvented in rabbits and we see no valid reason why it cannot be done in humans.

‡ The coagulation times in all instances were determined by the Lee-White modification of Howell's method. Gradwohl, R. B. H., *Clinical Laboratory Methods*, C. V. Mosby Co., St. Louis, 1935, p. 264.

13697 P

Inability of Purified Renin to Reduce the Blood Pressure of Hypertensive Dogs.*

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Wakerlin and his associates[1, 2] recently reported a striking reduction in the blood pressure of chronic, hypertensive dogs treated with daily injections of hog kidney extract over a period of 4 months. These authors believed the reduction in blood pressure to be closely related to a concomitant formation of an antirenin substance which they found in the blood of these animals.

They have not proved indubitably that the pressure reductions were due to the injection of the renin present in their renal extract, for this was comparatively crude, and contained many substances other than renin. These original observations have not yet been confirmed; furthermore, Winternitz and associates[3] could not obtain a reduction in pressure in their dogs following prolonged administration of crude kidney extracts or highly purified renin.

In the following preliminary report, results are given of the changes (a) in the blood pressure of 3 chronic, hypertensive dogs subjected to a prolonged course of daily, intramuscular injections of purified renin and (b) changes in the ability of the blood plasma of these dogs to neutralize the pressor effect of renin.

Methods. Two of the three hypertensive dogs used in this study (Dogs 13 and 28) were made hypertensive by application of a Goldblatt clamp[4] on the left renal artery and envelopment of the right kidney by cellophane.[5] The third animal (Dog 30) was made hypertensive by removal of the right kidney and envelopment of the left by cellophane. All of the dogs had been hypertensive for at least 54 days before renin injections were started.

* Aided by a grant from the Dazian Foundation for Medical Research.

Thanks are given to Wilfred Chew and Eleanor Williams for their technical assistance.

1 Johnson, C. A., and Wakerlin, G. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 277.

2 Wakerlin, G. F., and Johnson, C. A., *J. A. M. A.*, 1941, **117**, 416.

3 Winternitz, M. C., Mylon, E., and Katzenstein, R., *Yale J. Biol. and Med.*, 1941, **13**, 789.

4 Goldblatt, Harry, Lynch, J., Ranzal, R. F., and Summerville, W. W., *J. Exp. Med.*, 1934, **59**, 347.

5 Page, I. H., *J. A. M. A.*, 1939, **113**, 2046.

One day before the daily injections of renin were begun, plasma was obtained from each dog and tested for its ability to neutralize the pressor effect of renin in the manner described by Wakerlin.[2] These assays were repeated 6, 12, and 16 weeks after the beginning of renin administration.

Purified renin (Fraction D) was prepared from fresh hog kidney cortex according to the method of Helmer and Page.[6] Each dog received a daily, intramuscular injection of renin until he died (Dog 30), or until 4 months had elapsed. The dosage employed was equivalent to one gram of fresh kidney cortex per kilogram of body weight per day. Blood pressure recordings were taken thrice weekly by direct cannulation of the femoral artery.

Results. The continued daily injection of purified renin was not followed by a significant reduction in blood pressure (Table I). Dogs 18 and 28 had a reduction of 9 and 5% respectively after 123 days of daily renin injections. The blood pressure of Dog 30

TABLE I.

Effect of Daily Injections of Purified Renin upon Blood Pressure of Chronic Hypertensive Dogs.

	Average arterial blood pressure (mm of Hg)					
	Before renin injections		During renin injections			
Dog No.	Before hypertension*	After hypertension†	1st to 4th week‡	4th to 8th week‡	8th to 12th week‡	12th to 16th week‡
13	112	176	165	168	162	167
28	110	173	161	150	159	160
30§	124	163	176	190	226	

*Average blood pressure of week preceding the initial operation.

†Average blood pressure of 2 weeks preceding administration of daily renin injections.

‡Average blood pressure during 4-week interval.

§Died of uremia 74 days after daily renin injections were instituted.

TABLE II.

Pressor Effect of a Standard Renin Solution* when Injected into Anesthetized Nephrectomized Dogs Following Its Refrigeration with Plasma of Hypertensive Dogs Receiving Daily Injections of Renin.

	Maximum pressor response of nephrectomized dogs to refrigerated renin-plasma mixture			
Plasma used Dog No.	One day before renin injections commenced	During renin injections		
		End of 6th week	End of 12th week	End of 16th week
13	40	30	54	40
28	37	30	28	26
30	32	28		

*A single batch of purified renin, Fraction C, was used for test purposes. One cc of this solution was equivalent to 34.5 g of fresh hog kidney cortex.

[6] Helmer, O. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

actually increased during the renin administration (39% increase) although he died of uremia on the 74th day of treatment.

Following the prolonged administration of purified renin, the plasma of Dogs 13, 28, and 30 likewise showed no appreciable change in ability to neutralize the acute pressor effect of renin when refrigerated with it (Table II).

Discussion and Summary. The inability of prolonged administration of purified renin to change materially the blood pressure of the 3 hypertensive dogs reported herein does not necessarily refute the findings of Wakerlin, for he used a crude preparation of renin, and the reductions in pressure in his hypertensive dogs may have been due to some other factor present in his kidney extracts. The present observations indicate, however, that the prolonged administration of purified renin neither reduces the blood pressure of hypertensive dogs nor endows their plasma with the ability to inhibit the vasopressor response of renin.

13698

A Simplified Procedure for Preparing an Improved Pavlov Pouch.

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From the Department of Physiology of the Jefferson Medical College of Philadelphia.

The classical operation for preparing an innervated pouch ("Pavlov Pouch") of a portion of the stomach[1,2] is difficult and time-consuming and there is some doubt whether the pouch retains its full vagus innervation.[3] Modifications have been suggested which were designed to simplify the technic[4,5] or to preserve a larger proportion of the nerve supply.[6,7,8] However, with one exception[8]

1 Pavlov, I. P., *Ergebn. d. Physiol.*, 1902, **1**, 246.

2 Pavlov, I. P., *The Work of the Digestive Glands*, 2nd ed., London, 1910.

3 Jemerin, Edw. E., and Hollander, Franklin, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 139.

4 Boldyreff, W. N., *Bull. Battle Creek Sanit. and Hosp. Clinic*, 1925, **20**, 206.

5 Neuwelt, F., Olson, W. H., and Necheles, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 74.

6 Hollander, Franklin, and Jemerin, Edw. E., *Ibid.*, 1938, **39**, 87.

7 Cope, O., McMahon, C. E., Hagströmer, A., and Thompson, R. H., *Arch. Surg.*, 1940, **40**, 717.

8 Avrorov, P. P., and Schpuga, G. M., *Kab. Nauch. Meditsin. Untersuch.*, 1940, **12-13**, 238.

no procedure has been described which does not involve the complete separation of a part of the wall of the pouch from that of the main stomach with the consequent risk of severance of at least some of the nerve fibers.

The operation to be described leaves intact the entire muscular wall connecting the pouch with the main stomach. This has the double advantage of preserving the entire nerve supply and of avoiding the necessity of severing any of the larger blood vessels. Because no time is lost controlling hemorrhage, the operation can be performed more quickly, and therefore with less shock to the animal than the classical operation. This method exclusively has been used in our laboratory for the past several years with excellent results.

The general plan of the operation involves turning a portion of the stomach inside out through a small incision, thus making the mucosa accessible for the building of a mucosal wall between the projected pouch and the main stomach.

Detailed Procedure. The special instruments required are 2 pairs of vulsellum forceps and a set of gastroenterostomy forceps with 3 blades. The fundic portion of the stomach is delivered through an abdominal incision in the usual manner and the site of the pouch selected. An incision about two centimeters long is made in the center of the pouch area, through the entire gastric wall including the mucosa. This incision should be made parallel to the direction of the vagus fibers (see Jemerin and Hollander[3]), and not as indicated in the illustration.

Two pairs of vulsellum forceps are inserted through the gastric incision and the gastric wall grasped from the inside at the two points destined to be the extremities of the pouch (Fig. 1, A). By means of traction on the forceps and manipulation the entire pouch area is turned inside out through the incision.

One is now presented with a more or less circular rosette of mucosa with the vulsellum forceps attached to opposite points on its circumference. Further traction on the forceps, in opposite directions, pulls the circular rosette into an oblong shape so that two roughly parallel ridges of mucosa appear stretched between the forceps (Fig. 1, B).

The gastroenterostomy forceps are now applied so that the center jaw lies between the ridges of mucosa with its tip near the attachment of one pair of vulsellum forceps and as great a length as possible of each mucosal ridge is grasped between the center jaw and the corresponding outer jaw (Fig. 1, C). Even a large pair of forceps will be too small to encompass the whole distance between the pairs of vulsellum forceps. For this reason, when the procedures

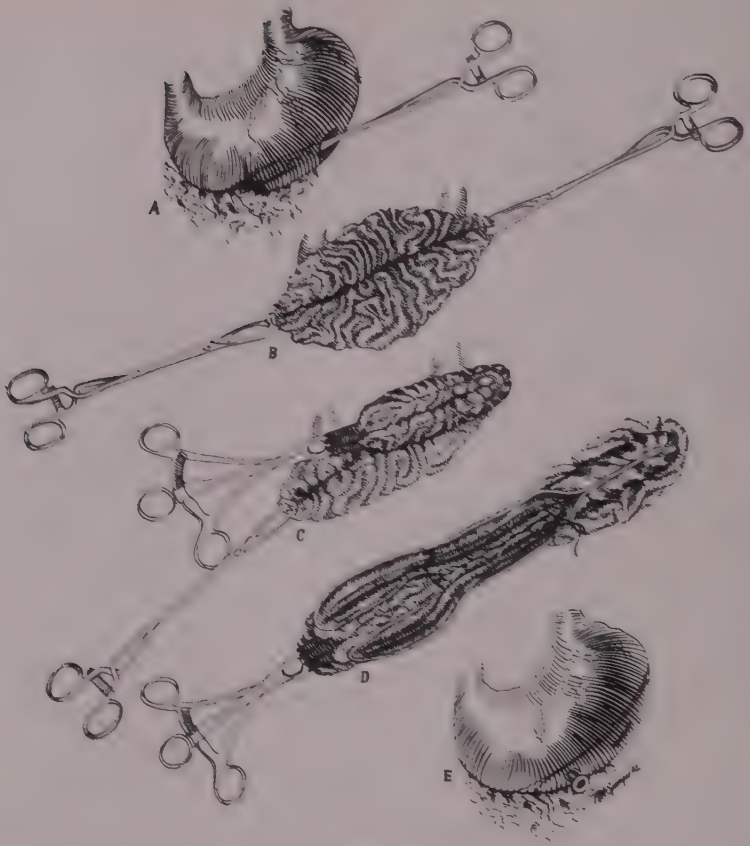

FIG. 1.

Various Stages of the Operation Described in the Text.

A. Vulsellum forceps attached to the gastric wall from the inside of the stomach preparatory to turning the pouch area inside out. A second pair of forceps (not illustrated) is attached at the upper end ("X") of the pouch area.

B. Appearance after turning the pouch area inside out. The darkly shaded region is the gastric lumen.

C. First position of the 3 bladed forceps. The upper end of the mucosal incision has been made and is to be continued along the dotted lines.

D. Diagrammatic representation of several steps showing the inner and outer suture lines and, at the lower left end, the mucosal flaps prepared for suturing.

E. The stomach with completed pouch. Better alignment of the cannula with the lumen of the pouch is obtained if the pouch runs somewhat from front to back rather than along the greater curvature as illustrated.

described below are completed in one area the gastroenterostomy forceps are shifted to the next adjacent area and so on until the entire distance has been covered. With the G.E. forceps in position a hairpin shaped incision is made through the mucosa only, extending along the crest of each ridge of mucosa and around the end where the

vulsellum forceps, which are now removed, were attached (Fig. 1, C). This incision is eventually to be extended so as to encircle the pouch area but at the moment is continued only to the point where the ridges of mucosa disappear between the blades of the gastroenterostomy forceps.

The next steps are illustrated by the composite diagram, Fig. 1, D. The mucosa along each side of the incision with some of the submucosa is dissected away from the remainder of the gastric wall, forming mucosal flaps about 1 cm in width (D, lower left end). The two inner flaps of mucosa are sutured together in such a way as to approximate the submucosal surfaces and turn the edges in toward the main stomach (D, midportion). The outer flaps are sutured in like manner but with the edges turned outward (D, upper right end). The suture lines are continued as far as possible, the inner a little farther than the outer, and the forceps are then shifted toward the other end of the pouch. The incisions and suture lines are continued as before, shifting the forceps whenever necessary until the suture lines are within a few centimeters of the second pair of vulsellum forceps which are still attached to the mucosa.

All the forceps are now removed and the incisions and suture lines continued until they meet where the ridges of mucosa join at the end of the pouch.

A double mucosal wall has been constructed between the pouch and the main stomach. The pouch is now turned right side out and the original small gastric incision closed with a double row of sutures. A hard rubber cannula is installed at one end of the pouch (Fig. 1, E) at the point judged to be the most dependent when the animal is in the upright position.* After the stomach has been returned to its natural position in the abdominal cavity the outer end of the cannula is brought out through a stab wound in the abdominal wall, so located as to cause the least displacement of the stomach. A soft rubber drain tube is inserted into the pouch *via* the cannula and held in place by means of a thread passed through small diametrical holes drilled through the cannula near its outer end.

From this point on the procedure, including after care, is the same as after any gastric operation. Dry food such as dog biscuit or dog "chow" is particularly to be avoided because it tends to cause rupture of the mucosal wall between the main stomach and the pouch.

* Dr. M. H. F. Friedman prefers to insert the cannula through the original gastric incision, in which case the incision should be made near one end rather than in the center of the pouch area.

13699

A New Method for Demonstrating Cyto-Antibodies *in vitro*.*

BJÖRN SIGURDSSON. (Introduced by A. Fischer.)

From the Biological Institute of The Carlsberg Foundation, Copenhagen.

As observed by Fischer[1] 2 pieces of tissue culture that have been excised and transferred to a test tube containing a physiological solution will coalesce in their growth and form one spherical block of tissue. The same will happen with small fragments of living tissue, for example, pieces from the heart of a young chicken embryo, if they are brought into contact with each other.

This fact may be used to demonstrate the presence of factors which will promote or prevent this coalescence, for example, various drugs and especially antibodies against tissue cells.

The technic employed was as follows: Hearts from embryos 8 to 10 days old were used. They were divided with a cataract-knife into about 50 pieces of approximately equal size. Two of these pieces were placed in each tube. Each piece measured about 0.5 mm in diameter (from 0.35 to 0.65 mm, a majority having a diameter of about 0.5 mm). The glass tubes measured about 8 mm in external and 6 mm in internal diameter. They were 4 cm in height, with a round bottom, provided with a small diverticulum, measuring about 2 to 3 mm in diameter and about 2 mm in depth. The tissue fragments were placed in this small receptacle to secure contact and to prevent their being separated by currents that might arise in the fluid. The tubes were stoppered with corks of proper size. The whole procedure was carried out with aseptic precautions.

The medium here employed was a mixture of equal parts of chicken serum and Ringer's solution. Only 2 or 3 drops are needed for each tube.

The tissue fragments were placed in the tubes with a platinum wire or the delicate point of a cataract-knife. Contact of the fragments was ascertained by means of a magnifying lens (the reverse ocular from a microscope). If they were not in contact, they could easily be moved by tilting and tapping the tube a little. The tubes were then incubated at 39° C, and readings were made after 6, 12, and 24 hours. When the 2 pieces formed one spherical body without

* This work was supported by the legacy of Consul General Ernst Carlsen and his wife.

1 Personal communication.

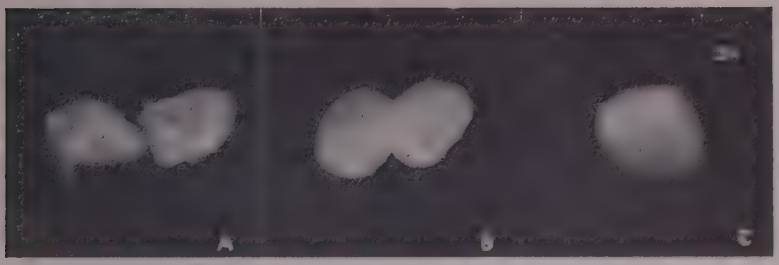

FIG. 1.

A. Tissue pieces before growth has begun. B. Coalescence is well under way (50%). C. Complete coalescence. The pieces were taken out of the tubes for better photographic reproduction.

any depression or indentation whatever it is designated as + or 100% coalescence. When there was substantial coalescence, but a definite groove could be discerned, where the 2 pieces met, it is designated as + —, or 66%. If the pieces were almost separate, but beginning to coalesce at a certain point, the result is called — +, or 33%. At this stage the pieces could not be separated by shaking the tube—a test that could be applied at the last reading only. With some experience in making the readings they may advantageously be graded more precisely, for example, in intermediate degrees too (Fig. 1).

In each experiment from 6 to 12 tubes were used in each series (experiments and control). Pieces from only one heart were used to exclude interference from individual differences in the hearts.

With this technic no visible reaction took place within 6 hours. After 12 hours they began to coalesce. After 24 to 48 hours they were partially or completely fused into one lump. The reaction is not very regular. Some of the tubes in a series may show + coalescence; others + — or — +; and some even —. As a rule the degree of coalescence after 24 hours estimated as described above is between 30 and 66%.

Simms and Stillmann[2] found that the latent period for growth *in vitro* of fibroblasts from the aorta of adult chickens was reduced by treating the tissue with "trypsin." Experiments with such a treatment were therefore carried out, in order to see whether it might influence favorably the inconsistency of my results and their reproducibility.

The "trypsin" used is a commercial preparation from Schering and Kahlbaum. A concentration of 0.2% in Tyrode's solution (pH

[2] Simms, H. S., and Stillmann, N. P., *J. Gen. Physiol.*, 1937, **20**, 603.

8.5 to 9) was used. The tissue fragments were placed in a water-bath at 37°C for from 15 minutes to 2 hours. They were then washed 3 times with Ringer's solution before they were put into the reaction tubes.

In Fig. 2 are shown the results from one of these experiments. The graphs are typical of a series of experiments carried out in the same way. There is a very pronounced difference between the "trypsin"-treated series and the untreated controls. In the former the coalescence has begun definitely within 6 hours and was completed, or almost completed, within 24 hours. Thus modified, the method yields results so uniform that it can be used to demonstrate the growth-inhibiting factors in a medium. It is evident that under the given experimental conditions a digestion period of 15 minutes only was sufficient to secure 100% coalescence in 24 hours. This finding does not permit the conclusion, however, that the optimal digestion period is 15 minutes. Treatment for 1 or 2 hours gave similar results.

A corresponding, preliminary incubation in Tyrode's solution alone did not bring about any difference in the percentage of coalescence.

As is well known, immune serum against tissue cells will inhibit the growth of the corresponding cells *in vitro*. (For references see

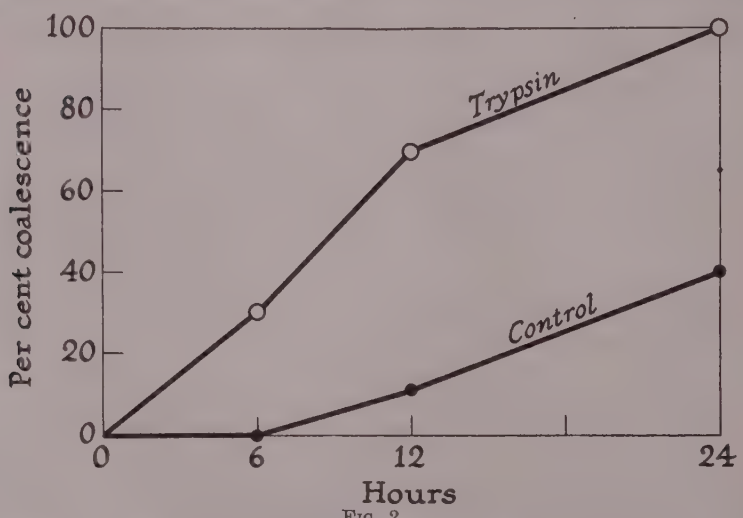

FIG. 2.

Shows the result of one experiment where the pieces in one series (marked Trypsin) were treated with "trypsin" for 15 minutes. The control series was treated with Tyrode's solution for the same length of time. For explanation of estimation of growth, see text.

Sigurdsson.[3]) Experiments were made to see whether the presence of such immune serum in the medium would prevent the coalescence of the tissue fragments. The immune serum produced in rabbits by injecting minced chick embryo inhibited the coalescence rather strongly, and in considerably lower concentrations than it inhibited growth in ordinary tissue cultures.[3] Normal rabbit serum did not have such an effect. The results from experiments with different concentrations of immune serum are shown in Fig. 3. It will be noted that immune serum in 10% concentration inhibits the coalescence completely; a concentration of 5% almost completely; and even 2% immune serum gives considerable inhibition.

This seems to be a simple and relatively reliable method for demonstration of cyto-antibodies and possibly other growth-inhibiting factors in a chosen medium.

Further it may be mentioned that heart fragments from 21-day-old chick embryos showed only a very poor tendency to coalesce. A brief treatment with "trypsin" (15 min.) alters this fact very little.

Many aspects of the method need further investigation: for example, the optimal intensity of "trypsin" treatment, the possible influence of embryonal extract in the medium, its applicability to pharmacological purposes, etc., but the work had to be discontinued.

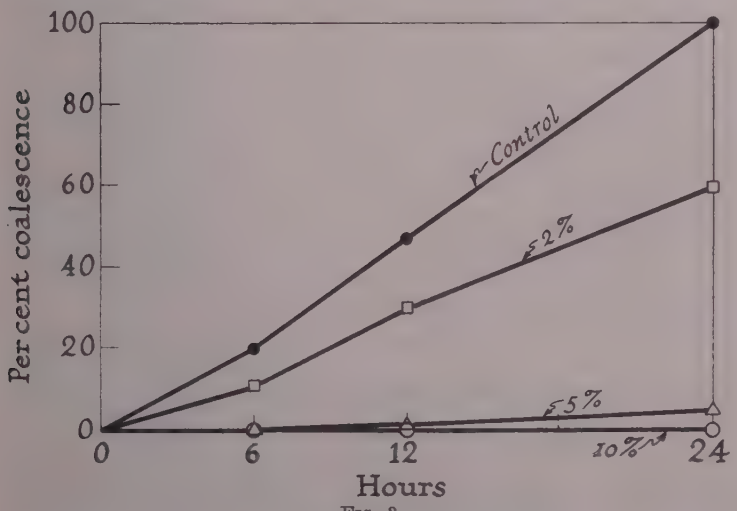

FIG. 3.

Rabbit immune serum against chicken tissue was added to the medium in the percentage indicated. All the tissue pieces had been treated with "trypsin." Control does not contain antiserum.

[3] Sigurdsson, B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 237.

Summary. Small pieces of heart tissue from young chick embryos coalesce to form a spherical body when brought into actual contact with each other in a mixture of chicken serum and Ringer's solution. The pieces must touch each other. A preliminary treatment of these fragments with "trypsin" increases the rate and percentage of coalescence. The method is serviceable for titration of cyto-antibodies in a medium, as these inhibit the coalescence in low concentrations.

The method is described, and its use is demonstrated by examples.

13700

Effect of a Single Dose of 3,3'-Methylenebis (4-Hydroxycoumarin) upon Blood Coagulation in Humans.*†

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The pharmacological and chemical activity of 3,3'-methylenebis (4-hydroxycoumarin) has been reported from agricultural experiment stations.[1,2] The most recent publication[3] by the Wisconsin workers describes the effect of a single dose of this dicoumarin upon the prothrombin time of the plasma of various animals. In this communication we describe the effect of single doses of this drug on blood coagulation in humans. Prandoni and Wright[6] have reported on the effect of daily doses in man. However, the few re-

* Technical assistance was rendered by Miss Allene Graves and Miss Adine Levy.

† These studies received additional support from the Metropolitan Life Insurance Company and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch and Mrs. H. Robert Samstag.

[1] Roderick, L. M., and Schalk, A. F., *North Dakota Agr. Exp. Sta. Bull.*, 1931, 250.

[2] Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

[3] Overman, R. S., Sturmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

[4] Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sc.*, 1941, **202**, 568.

[5] Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet. Mayo Clinic*, 1941, **16**, 388.

[6] Prandoni, A. G., and Wright, I. S., presented before the N. Y. section of the Soc. for Exp. Biol and Med., Nov. 20, 1941.

ported instances[4, 5] of the use of 3,3′-methylenebis (4-hydroxycoumarin) in man have not yet provided enough information to warrant its general use.

Materials and Methods. Weighed doses of 3,3′-methylenebis (4-hydroxycoumarin)‡ were administered orally with water. Thirty-nine patients ranging from 12 to 70 years of age with most between 40 to 60 years, were selected for study. There were 21 with arteriosclerotic heart disease and a varying amount of cardiac failure, 9 with pneumonia, 5 convalescing from fracture of an extremity and others. The pneumonia patients were studied early in the course of their disease when the temperature was elevated and they were acutely ill. The weights of the patients varied from 100 to 180 lb, the mean being 140 lb.

Forty-five single doses of this dicoumarin were given. Prothrombin and coagulation times were performed on alternate days or more frequently. There were 5 doses of 100 mg or less, 5 of 200 mg, 6 of 300 mg, 10 of 400 mg, 11 of 500 mg, 4 of 600 mg, and 4 of 700 mg. The per kilo dose averaged 8 mg, varying from 1 mg per kilo to 12 mg per kilo. The patients were studied from 4 to 24 days after the drug was given; most were followed for 10 days.

Before the drug was administered 7 cc of venous blood was drawn with minimal stasis in a syringe and needle previously rinsed in 0.85% saline; 4.5 cc of blood was immediately mixed with 0.5 cc of 0.1 M sodium oxalate; the coagulation time was determined at room temperature with the remainder by a minor modification of the two-tube method of Lee and White.[7] All tubes used in this study were cleaned in green soap and cleaning solution, adequately rinsed and oven-dried. The oxalated blood was centrifuged several hours after collection and the plasma stored at 6°C. The patients were seen daily and examined for possible toxic effects of the drug.

The prothrombin determinations were performed by a modified Quick procedure[8] using acetone-treated rabbit brain as a source of thromboplastin and recalcifying the plasma and thromboplastin mixture with 0.0125 M calcium chloride. The brains were obtained from discarded Friedman pregnancy-test rabbits.

The reaction was carried out at 37°C with plasma diluted with normal saline to from 62.5% to 25% concentration. Duplicates gave close agreement. Normal plasma was used as a control whenever test plasma was titrated. The final result for

‡ Supplied by Dr. J. F. Biehn of Abbott Laboratories, Inc., North Chicago, Ill.

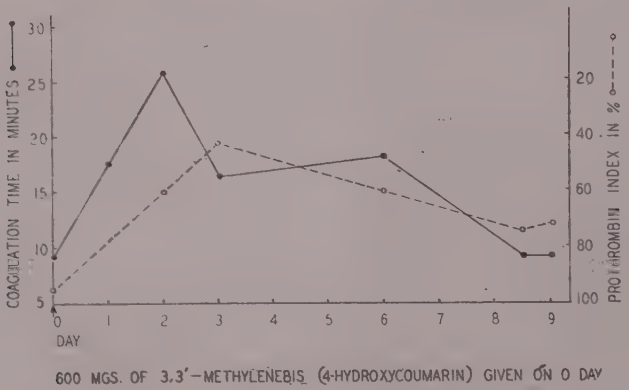
[7] Lee, R. I., and White, P. D., *Am. J. Med. Sc.*, 1913, **145**, 495.

[8] Quick, A. J., *J. Am. Med. Assn.*, 1938, **110**, 1658.

any dilution of plasma was expressed as: prothrombin index in $\% = 100 \left(\frac{\text{control plasma in sec.}}{\text{test plasma in sec.}}\right)$. Quick[8] and Pohle and Stewart[9] and others reported normal human prothrombin time of undiluted plasma of between 10 and 13 seconds. Our average undiluted plasma prothrombin time varied between 14 and 20 seconds.

Results. Graphs 1 and 2 represent the effect of this dicoumarin on the coagulation time and prothrombin index of 2 different patients. The first detectable change in the coagulation mechanism occurred in from 24 to 72 hours after oral administration of 3,3′-methylenebis (4-hydroxycoumarin) and was manifest by a prolongation of the coagulation time or a fall in the prothrombin index. Though in most cases these changes occurred together, sometimes they were dissociated. The maximum effect from the drug was usually observed 1 to 3 days after the commencement of action, but this varied. Elevation of the coagulation time was quite variably maintained in different individuals and was unrelated to the dose. From a single dose, the duration of effect varied from 3 to 15 days averaging 6 days. The return of the clotting time to normal occurred either suddenly or gradually over a period of several days. It was unusual for a plateau to be reached in which the coagulation time remained evenly elevated and without considerable swings. As normal values were resumed, the coagulation time and prothrombin index were usually parallel but there were instances in which one became normal while the other still remained abnormal.

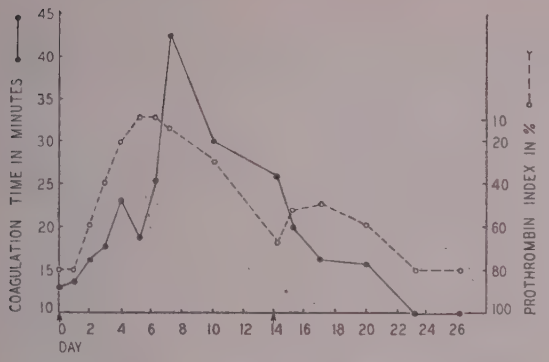
From these data it appears that the oral threshold dose of 3,3′-



GRAPH 1.

Effect of a single dose of 3,3′-methylenebis (4-hydroxycoumarin) upon coagulation time and prothrombin index of blood.

9 Pohle, F. J., and Stewart, J. K., *Am. J. Med. Sc.*, 1939, **198**, 622.



GRAPH 2.

Effect of single doses of 3,3′-methylenebis (4-hydroxycoumarin) upon coagulation time and prothrombin index of blood.

methylenebis (4-hydroxycoumarin) for adults is approximately 400 mg. Only one patient who received this amount failed to show the characteristic response. It is probable that 400 to 600 mg of this dicoumarin orally is the dose most likely to produce desired prolongation of clotting time safely. All cases receiving more than 5 mg per kilo of body weight showed a lowering of the prothrombin index and an elevation of the coagulation time.

In this study the initial control coagulation times before administration of 3,3′-methylenebis (4-hydroxycoumarin) varied between 6 and 19 minutes, averaging 11 minutes. At the height of drug action, the coagulation time varied between 16 and 42 minutes; most cases were between 22 and 26 minutes. All the pretreatment prothrombin indices ranged between 80% and 100%; during drug action they varied from 100% to less than 10% of normal.

When given in a single dose, there were practically no toxic manifestations. Two patients had minor epistaxes at a time when their coagulative mechanism was slightly abnormal. Both had been subject to such episodes prior to admission. The bleeding stopped spontaneously within one-half hour. It is difficult to interpret the evidence of toxicity in a third patient who entered in an advanced degree of cardiac failure and received 700 mg. Autopsy was performed and revealed, besides evidence of marked congestion due to heart failure, an unusual edema and intense hyperemia of the bronchi. There was a large quantity of hemorrhagic fluid in the bronchi. Three other patients who died of carcinomatosis or tuberculosis during or shortly after study showed no lesion that could

be interpreted as due to the dicoumarin administration. This pathological material is still under investigation. We have administered the drug to 20 other patients not included in this series and have not observed bleeding or other manifestations of toxicity. However, bleeding has been observed after the first week of major surgical procedures, as when a packing is removed. On this account, dicoumarin should be administered with caution to such patients.

Discussion. These observations indicate that the action of a single dose of 3,3′-methylenebis (4-hydroxycoumarin) is prolonged, and suggest the possibility that a few properly spaced doses may be sufficient to lengthen the coagulation time in humans for 2 weeks or longer. The therapeutic possibilities of this procedure are being investigated. During the latent period, 1 to 3 days before the action of the drug is apparent, heparin by continuous intravenous drip may be used. The heparin is discontinued as soon as the dicoumarin effect is seen. Further experimentation with these drugs in humans is desirable before they can be recommended for general use in thromboembolic states.

Summary. A prolongation in the coagulation time and a lowering of the prothrombin index of the blood for approximately 6 days beginning between 24 and 72 hours after the drug has been ingested will usually be produced by oral administration of a single dose of 400 mg of 3,3′-methylenebis (4-hydroxycoumarin) to a patient weighing 130 lb or less, or 600 mg to a patient weighing over 160 lb.

13701

Toxic Effects of Some Basic Proteins.

L. REINER, E. J. DEBEER AND MILTON GREEN.

From The Burroughs Wellcome & Co. U. S. A. Experimental Research Laboratories, Tuckahoe, N.Y.

Proteins which have an isoelectric point on the basic side of neutrality (protamine, histone), or near neutrality (globin) have recently been used in combination with insulin as depository therapeutic preparations. Their toxicities, however, have not been extensively studied. Vartiainen and Marble[1] reviewed the literature on the toxicity of protamine and determined its acute toxicity by

[1] Vartiainen, I., and Marble, A., *J. Lab. Clin. Med.*, 1941, **26**, 1416.

subcutaneous injection in mice and rabbits and found the LD 50 to be about 250 mg kg. Kossel[2] notes that the toxicity of histones is similar to that of protamines. Thompson[3] studied the pharmacology of protamines and thymus histone and Annau, *et al.*,[4, 5] studied more extensively the pharmacology of histone prepared from avian red cells. The action was found to be similar to that of shock-producing substances. Obviously, the acute toxic dose of these proteins is much larger than their average daily doses given together with insulin. Local reactions, however, are not infrequent after the injection of protamine zinc insulin, but they have been attributed to sensitivity to protamine.

In view of the wide and repeated application of these proteins, it seemed of interest to investigate their general and tissue toxicity.

Acute Toxicity. The monoprotamine sulfate solutions used contained 5 mg cc and were injected intraperitoneally into mice in graded doses. Deaths occurred within a few hours. The symptoms were depression, cyanosis, and short, convulsive hops. The LD 50 was 94 mg kg. The slope of the regression line, when the logarithm of the dose was plotted against probit units, was 9.

Histone sulfate was injected intraperitoneally in a solution containing 20 mg cc, using graded doses. Deaths occurred within 2 days. The symptoms were depression followed by coma. The LD 50 was 255 mg kg. The slope of the regression line was 12.5.

Globin was injected as a solution containing 30 mg of globin per cc. One cc per mouse was well tolerated by all 10 mice used.

Tissue Toxicity. In order to obtain some evidence with regard to the toxicity of these proteins to mammalian tissue, the oxygen consumption of minced rat liver was determined in buffer solutions (0.10 molar phosphate, pH 7.3) containing 0.3% glucose and 10% fresh rat serum. To 1 cc of liver suspension (.25 mg dried liver) in this medium, 0.35 cc of 1.7% of the various protein solutions was added from the side arm, after a short initial period of respiration. The oxygen consumption was measured in the Barcroft-Warburg vessel at 37°C with air as the gas mixture.

The relative rates of oxygen consumption are illustrated in Graph 1. Monoprotamine sulfate and histone sulfate showed a definite inhibition of the oxygen consumption, whereas globin did not.

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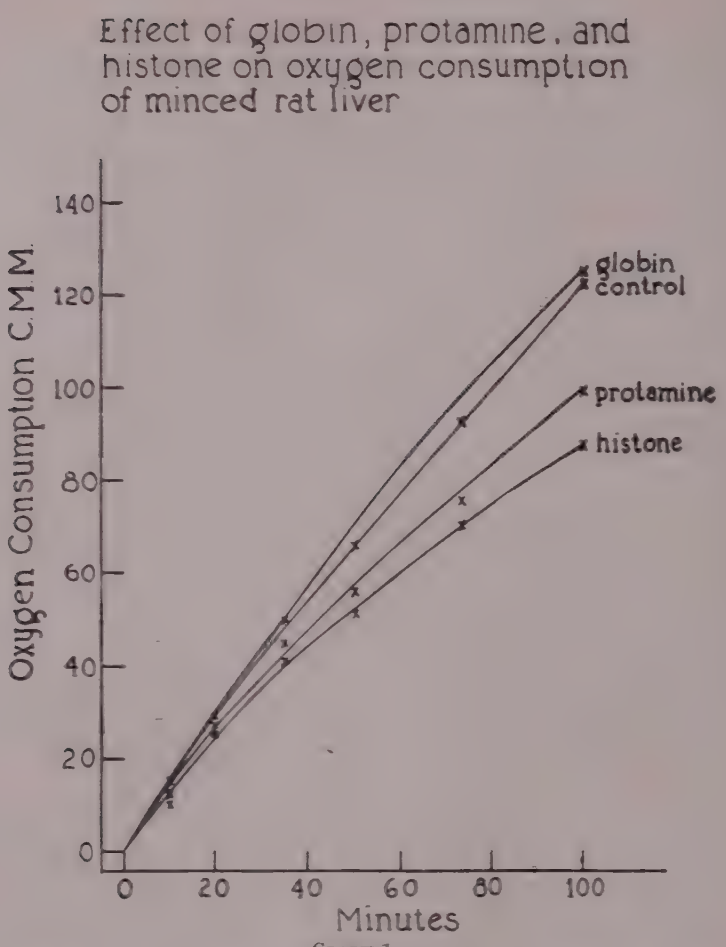
2 Kossel, A., *The Protamines and Histones*, Longmans, Green and Co., Ltd., 1928.

3 Thompson, W., *Z. physiol. Chem.*, 1900, **29**, 1.

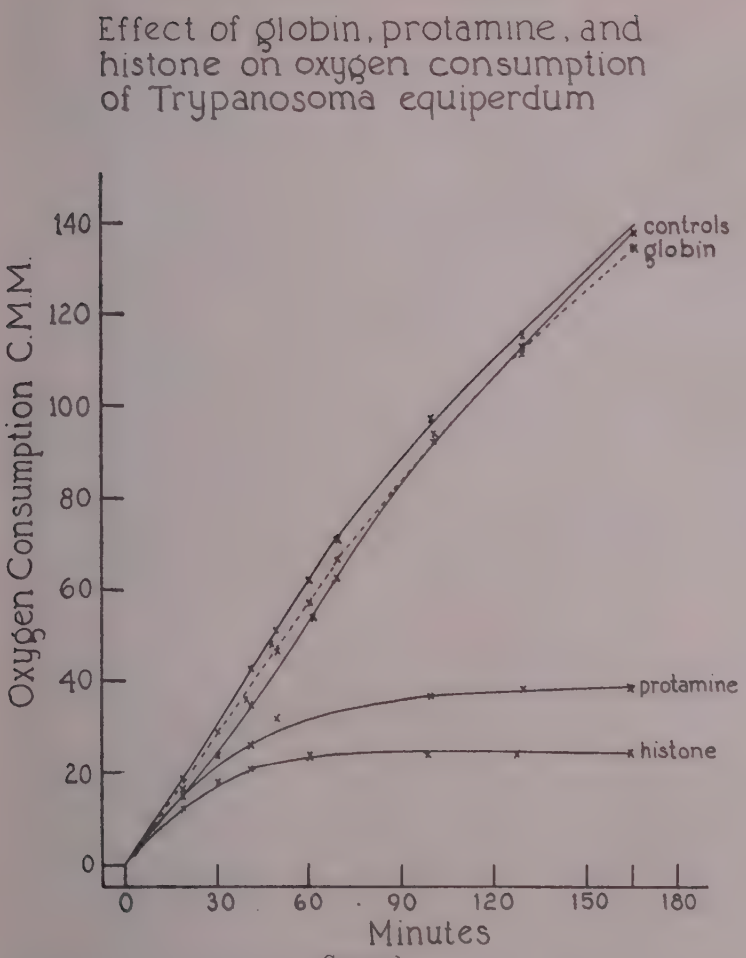
4 Annau, E., and Augustin, V., *Arch. exp. Path. Pharm.*, 1931, **161**, 337.

5 Annau, E., and Huszák, I., *Arch. exp. Path. Pharm.*, 1932, **163**, 541.



GRAPH 1.

Toxicity to Trypanosomes. *Trypanosoma equiperdum*, obtained from infected rats by fractional centrifugation of the blood, were suspended in a medium consisting of 10 parts of 0.11 molar phosphate buffer, pH 7.3, 9 parts of 0.85% sodium chloride, and 1 part of 6% glucose. The suspension contained 4×10^7 trypanosomes per cc. 0.2 cc of the 1.7% protein solutions was added from the side arm, but otherwise experimental conditions were the same as above. The results given in Graph 2 show that both monoprotamine sulfate and histone sulfate were more toxic to trypanosomes than to rat liver, and that no toxic effects were observed when globin was added.



GRAPH 2.

Discussion. Our findings confirm in general the qualitative information discussed by Kossel. These results can be considered as evidence from a physiologic point of view for the necessity of distinguishing globins, histones, and protamines from each other.

It is of interest to note in this connection that insulin was found to be well precipitated by various capillary-active, strongly basic compounds, among others, by the quaternary ammonium salt type of disinfectant.[6] We now find that proteins containing a relatively large amount of basic amino acids with a long chain such as arginine and lysine are toxic to tissues and to trypanosomes. Bacteriostatic

[6] Lang, E. H., Buck, J. S., and Reiner, L., *Pharm. Arch.*, 1941, **12**, 81.

activity of protamines was also observed by McClean.[7] Annau, *et al.*, believe that the pharmacological effects of histones are associated with the arginine radical present in the molecule in a peptide linkage. Free arginine having a free carboxyl group is entirely ineffective pharmacologically. It seems probable that the presence of arginine radicals in peptide combination conveys properties to the histone and protamine molecules which are to some extent similar to those of cationic detergents. The mechanism by which these substances exert their toxic effects on cells is not clearly understood. Whether or not the reactivity of these substances with insulin can be taken as suggestive of the mechanism of their toxic action remains to be investigated.

Summary. The order of the toxicity of the proteins investigated is as follows: protamine > histone > globin. Globin was found to be devoid of appreciable toxicity when injected into animals or tested on tissues and on monocellular organisms *in vitro*. Protamine and histone were found to be highly toxic to trypanosomes. Factors which may contribute to the toxicity of these proteins were discussed.

13702

Origin of Ionized Iron after Action of Acids on Blood and Influence of Carbon Monoxide.

GEORG BARKAN* AND OTTO SCHALES. (Introduced by F. H. Pratt.)

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When dilute acids are added to hemolyzed blood, a small amount of iron is split off in ionized form,[1-4] varying from 14 to 18 mg per liter of human blood.[5, 6, 7] It was assumed that this easily split-off

7 McClean, F., *J. Path. Bact.*, 1931, **34**, 459.

* Partially aided by the Rockefeller Foundation.

We gratefully acknowledge the assistance of the Ella Sachs Plotz Foundation in furtherance of our work.

1 Barkan, G., *Z. physiol. Chem.*, 1925, **148**, 124.

2 Lintzel, W., *Z. Biol.*, 1925, **83**, 289.

3 Barkan, G., *Z. physiol. Chem.*, 1927, **171**, 179.

4 Barkan, G., *Z. physiol. Chem.* 1927, **171**, 194.

5 Schwarz, L., and Deckert, W., *Klin. Woch.*, 1935, **14**, 601, 900.

6 Olesk, J., *Klin. Woch.*, 1935, **14**, 1006.

7 Rooks, G., *Deutsch. Z. gerichtl. Med.*, 1936, **27**, 47.

iron was due to the presence in the erythrocytes of iron-containing bile pigment proteids (pseudohemoglobins[8, 9]). The presence of non-hemoglobin iron in the erythrocytes has been confirmed by a number of recent investigators, using different methods.[10–19] In the above experiments,[1, 3–9] the determination of the liberated iron was carried out in protein-free filtrates obtained from hemolyzed blood solutions, which had been incubated at 37°C for 24 hours with 0.4% HCl. The low concentration of acid was considered necessary in order to avoid any attack on the iron in the hemoglobin molecule itself. Some recent data[18, 20] have shown, however, that incubation with HCl in concentrations lower than 0.4% results in even higher values for the easily split-off iron.

These observations and other reports[21, 22] make it doubtful that the values for the easily split-off iron obtained with the customary method[23–25] give a true picture of the amount of pseudohemoglobins originally present in the blood.

The purpose of the present investigation was to determine whether labile iron can originate from the action of dilute acid on hemoglobin[2, 22] and if so, whether it is possible to differentiate between the liberation of iron from hemoglobin and from preformed pseudohemoglobin.

Action of acids on native and synthetic hemoglobin. The effect of various concentrations of acid on solutions of human blood was studied. Venndt[20] had found that 1.3% HCl solution liberated less and 0.1% HCl solution liberated more iron than did 0.4% HCl solu-

8 Barkan, G., and Schales, O., *Z. physiol. Chem.*, 1937, **248**, 96.

9 Barkan, G., and Schales, O., *Z. physiol. Chem.*, 1938, **253**, 83.

10 Starkenstein, E., and Weden, H., *Arch. exp. Path. u. Pharm.*, 1928, **134**, 278.

11 Dominici, G., *Arch. per le sc. med.*, 1929, **53**, 538.

12 Winegarden, H. M., and Borsook, H., *J. Cell. and Comp. Physiol.*, 1933, **3**, 437.

13 Bansi, H. W., and Rohrlich, M., *Verh. Deutsch. Gesellsch. inn. Med. XLV Kongr.*, 1933, p. 350.

14 Tompsett, S. L., *Biochem. J.*, 1934, **28**, 1536.

15 Klumpp, Th. G., *J. Clin. Invest.*, 1935, **14**, 351.

16 Josephs, H. W., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 50.

17 Shorland, F. B., and Wall, E. M., *Biochem. J.*, 1936, **30**, 1049.

18 Moore, C. V., *J. Clin. Invest.*, 1937, **16**, 613.

19 Jenkins, C. E., and Thomson, M. L., *Brit. J. exp. Path.*, 1937, **18**, 175.

20 Venndt, H., *Z. physiol. Chem.*, 1940, **263**, 162.

21 Miller, L. L., and Hahn, P. F., *J. Biol. Chem.*, 1940, **134**, 585.

22 Legge, J. W., and Lemberg, R., *Biochem. J.*, 1941, **35**, 353.

23 Barkan, G., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Berlin and Vienna, 1935, 7 Abt. V, Teil 8, 1207.

24 Barkan, G., *Klin. Woch.*, 1937, **16**, 300.

25 Barkan, G., and Walker, B. S., *J. Biol. Chem.*, 1940, **135**, 37.

TABLE I.

HCl%	12	6	4	3	2	1	0.4	0.12	0.10	0.05
Fe mg/liter blood sol. 1:10	1.03	0.92	1.13	1.32	1.25	1.56	1.84	4.50	4.89	2.11

tion. These results have been confirmed and extended. A series of iron determinations on diluted blood (1:10) incubated for 48 hours at 37° C. in HCl solutions varying from 12 to 0.05%, was done. Table I shows an example with human blood containing 15.63 g Hb per 100 ml; "Liquoid" (Roche) was used as an anticoagulant.

An incubation time of 48 hours was chosen in order to secure a more complete reaction.[29] The iron determinations were carried out electrophotometrically with o-phenanthroline.[25] A correction had to be made for the brownish color of the protein-free filtrates obtained from mixtures with HCl above 2%.

The table shows no significant change in the amount of iron split off with concentrations of HCl between 12 and 2%, but there is a definite increase below this concentration with a sharp peak at about 0.1% HCl. The amount of iron given off in this range of low acid concentrations represents about 10% of the total blood iron. Such an amount of non-hemoglobin iron is not in accordance with known facts, and at least in this range a considerable part of the iron found originates from the hemoglobin molecule itself.

For a dependence of the breakdown of hemoglobin on the acidity of the solution, an explanation can be given. On the addition of acid to oxyhemoglobin solutions active oxygen is liberated instantly,[26, 27] which probably then forms hydrogen peroxide.[28] At low acid concentrations a considerable amount of pigment with intact protein-hemin linkage will exist for a time and this acts as a peroxidase, catalyzing the oxidative self-destruction of a part of the pigment with the formation of open-ring compounds with labile iron. At higher acid concentrations, however, the protein moiety of hemoglobin will be attacked immediately and hemin is set free. This is a much less active peroxidase than hemoglobin, but has about the same catalatic activity[25] as the intact blood pigment. Consequently at higher acid concentrations any hydrogen peroxide formed will be destroyed rather than utilized for the destruction of blood pigment.

[26] Lemberg, R., and Legge, J., *J. and Proc. Roy. Soc. New South Wales*, 1938, **72**, 62.

[27] Fujita, A., Ebihara, T., and Numata, I., *Biochem. Z.*, 1939, **301**, 245.

[28] Lipschitz, W., in *Handbuch d. norm. und pathol. Physiol.*, Berlin, 1928, VI/I, 149.

[29] Kuhn, R., and Brann, L., *Ber. chem. Ges.*, 1926, **59**, 2370.

TABLE II.

Material	Total iron mg/liter	Iron split off by HCl 0.4% (48 hrs at 37°C) mg/liter	Iron split off by HCl 0.1% (48 hrs at 37°C) mg/liter
"Synthetic" methemoglobin (2.4 g/100 ml)	80.3	0.23	0.25
Blood solution (3.13 g Hb/100 ml)	105.0	3.68	9.78

(The results for "synthetic" methemoglobin are corrected for the Fe content of the globin solutions from which it was prepared.)

For an iron-containing bile pigment-globin compound (pseudohemoglobin) such a dependence of the liberation of its iron on the acid concentration is improbable. The results obtained with 2-12% HCl then would represent the amount of iron derived from preformed pseudohemoglobins, equal to only about 2% of the total iron. This would agree in the order of magnitude with the amount of bile pigments isolated by Lemberg and associates[30] from red blood cells.

In contrast to the experiments of Legge and Lemberg,[22] methemoglobin prepared by combining native bovine globin[32] with crystalline chlorhemin,[32] did not split off more than 0.3% of its total iron under conditions in which the yield of liberated iron from blood solutions is highest (Table II).

As methemoglobin in contrast to oxyhemoglobin does not form active oxygen or hydrogen peroxide on addition of acid, a destruction similar to that suggested for oxyhemoglobin in 0.1% HCl will not take place.

Influence of carbon monoxide. When blood solutions are treated with CO before adding HCl, a much smaller amount of iron is split off.[33] A blood solution saturated with CO and incubated in 0.4% HCl yields only about ⅓ of the amount of iron liberated without carbon monoxide treatment. With amounts of CO not sufficient

TABLE III.

Human blood, Hb 16.7 g. 100 ml. Dilution 1:10 digested 24 hrs with 0.4% HCl at 37°C.

Fraction of total Hb present as HbCO (determined spectrophotometrically) %	Iron split off mg/liter original blood	Amount inhibited	Inhibition in % of that in sol. saturated with CO
0	17.1	0	0
36	9.6	7.5	65
71	6.3	10.8	94
100	5.6	11.5	100

30 Lemberg, R., Lockwood, W. H., and Legge, J. W., *Biochem. J.*, 1941, **35**, 363.

31 Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1929-30, **13**, 469.

32 Nencki, M., and Zaleski, J., *Z. physiol. Chem.*, 1900, **30**, 390.

33 Barkan, G., and Berger, E., *Arch. exp. Path. u. Pharmakol.*, 1928, **136**, 278.

TABLE IV.

No.	Incubation time, hr	Iron split off by 0.4% HCl from sol. saturated with O_2 (mg/liter original blood)	Iron split off by 0.4% HCl from sol. saturated with CO (mg/liter original blood)	Iron split off by 0.4% HCl from sol. saturated with CO and reduced with $Na_2S_2O_4$ (mg/liter original blood)	$Na_2S_2O_4$ added per ml blood sol. 1:5, mg
1	24	16.8	2.8	1.4	.50
2	24	16.8	5.6	3.5	.33
3	48	22.0	6.5	2.7	.10

for complete transformation of HbO_2 into HbCO, the inhibition of the liberation of iron found is not proportional to the percentage of HbCO (see example in Table III).

The amount of iron split off becomes still smaller, if the solutions, after treatment with CO, are reduced and again treated with carbon monoxide. Table IV gives the results of some experiments of this type carried out with solutions (1:10) of defibrinated beef blood.

While the phenomena here involved are more complicated than previously assumed,[8, 22] the fact remains that the decrease by CO of the lability of iron from blood solutions gives a means of detecting smaller amounts of carbon monoxide in blood than are determinable by simple qualitative tests for HbCO. In 80 cases of carbon monoxide poisoning, Schwarz and Deckert[5] confirmed Barkan and Berger's[33] findings and noted that the decrease in labile iron would persist for several days after the acute exposure to carbon monoxide. Rooks[7] found the method valuable also for *post mortem* diagnosis of CO-poisoning. Schwarz and Deckert noticed that less labile iron was found in the blood of habitual smokers. With some reserve this was referred to as a carbon monoxide effect. Schwarz and Deckert's observation was confirmed by Olesk[6] and by Rooks.[7] These findings have also been confirmed here, as Table V shows. Twenty-four hours' digestion with 0.4% HCl and the photoelectric method for iron determination[25] were used.

Summary. 1. The amount of labile iron derived from preformed

TABLE V.

Group	No. of persons	Hemoglobin, avg, g/100 ml	Easily split-off iron, avg, mg/liter	Standard error of the mean, ±
Non-smokers	10	14.8	14.2	0.37
Smokers*	12	14.8	12.8†	0.34

*10-30 cigarettes a day.

†According to R. A. Fisher, *Statistical Methods for Research Workers*, 4th ed., the difference is significant. The probability for chance is about 0.0125 (see Table IV, table of t, *l.c.*).

pseudohemoglobins is not more than 2% of the total blood iron. 2. Under the influence of very dilute acids additional iron is split from the hemoglobin molecule itself. 3. Carbon monoxide inhibits the liberation of iron. 4. This inhibitory effect can be used for diagnostic purposes and confirms the presence of carbon monoxide in the blood of tobacco smokers.

13703

Permanent Devocalization of Dogs by Removal of both the True and the False Vocal Cords.

G. L. DONNELLY. (Introduced by W. deB. MacNider.)

From the Laboratory of Pharmacology, the Medical School, the University of North Carolina.

In many institutions where it has been necessary to devocalize the laboratory dogs the results have not been satisfactory. In 1932 the writer made a study of the usual devocalizing methods with the object in view of improving the results obtained from such methods.

By using a pair of Miles nasal cutting forceps it was found that the true vocal cords could be rapidly and completely removed.[1] The procedure was carried out on 20 dogs under morphine-ether anesthesia. There were no complications and the animals recovered within 24 hours. After about 3 months, however, each dog developed a hoarse, unpleasant bark. The immediate assumption was that the operative technic had been faulty and that the vocal cords had not been completely removed. Later, when some of the same animals came to autopsy, laryngeal examinations showed a complete absence of the true vocal cords. Further studies of the autopsy material revealed in each case a well developed pair of soft mucous folds or pockets at the base of the epiglottis and just above the true vocal cords. These folds were identified as false vocal cords and seemed to be the only remaining structures by the use of which sound might be made.

The surviving dogs whose vocal cords had been removed with poor results were again prepared for operation. The soft folds or pockets of the false cords were removed as completely as possible with a pair of straight, back-cutting Ostrom forceps. Upon recovery these animals were no longer able to produce any noise other than

[1] Andrews, Justin M., *Science*, 1926, **64**, 502.

soft whispered sounds even with their most vigorous barking efforts. Since that time approximately 200 animals have been devocalized by the method of removing both the true and false vocal cords. Animals still living 8 and 9 years after the operation have had no return of vocal powers. It is probably worth noting that they seem to derive as much pleasure from the whispered bark as from the noisy one.

The following is a brief description of the procedure. The animal is given 0.25 cc per kilo of a 2% solution of morphine sulfate with 0.5 mg atropine sulfate subcutaneously from one to 2 hours before the operation. It is then tied to the operating table back down but with head dependent. Following the induction of complete ether anesthesia the mouth is held open by an assistant who pulls out on the tongue with a pair of Allis tissue forceps. Proper illumination is obtained with a head light. The operator by pulling the epiglottis forward with a pair of Tydings tissue forceps is able to visualize the vocal cords clearly. When both true vocal cords have been removed and no tags of tissue remain, the epiglottis is again pulled forward and the operator inserts the hinged tip of the Ostrom forceps under the false cord. The fold or pocket is removed as completely as possible and the procedure is then repeated on the other side. Due to the soft, stringy nature of the tissue in these cords the cutting is more difficult and the bleeding is more profuse than with the true cords. Following operation the animal is laid in the cage with head slightly lowered to prevent aspiration of blood. In well nourished animals, however, the danger of complications is small.

In conclusion, the author has found from his own experience and from the experience of others that no matter how completely the true vocal cords are removed the dogs learn to bark again. If, however, both true and false vocal cords are removed the dogs remain completely and permanently devocalized.

13704

Method for Determining Completeness of Thyroidectomy Using Radioactive Iodine.*

WILLIAM O. REINHARDT. (Introduced by Herbert M. Evans.)

From the Division of Anatomy and Institute of Experimental Biology, University of California.

It has been shown that radioactive iodine injected into or ingested by experimental animals is concentrated in the thyroid gland, and that it is possible to obtain evidence of its presence using either radioautographic[1, 2] or electroscopic methods.[3] It was thought reasonable to attempt to determine the presence or absence of thyroid tissue in thyroidectomized animals employing a sensitive indicator such as the X-ray film. The only valid method for determining completeness of thyroidectomy at the present time consists in producing complete serial sections of the neck region. In fact, in order to prove completeness of thyroidectomy, it is necessary to section the entire path of development of the thyroid gland from the base of the tongue down to and including trachea, thymus, great vessels and heart. Serial sectioning of this amount of tissue involves a disproportionate amount of time and labor.

To test the validity of a search for thyroid tissue using radioactive iodine, the following experiment was carried out. Twelve female rats of the Long-Evans strain thyroidectomized at the age of 70 days were injected at the age of 133 days with a solution containing radioactive iodine.† A normal, unoperated animal was included for purposes of control. Oxygen consumption tests were carried out shortly before autopsy.‡ In those cases in which the oxygen consumption was greater than that expected of a completely thyroidectomized rat, the determinations were repeated. The lower figure is reported in each case.

The method employed for obtaining the radioautographs was as

* Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation, New York City.

1 Hamilton, J. G., Soley, M. H., and Eichorn, K. B., *Univ. of Calif. Publ. Pharm.*, 1940, **1**, 339.

2 Gorbman, A., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 103.

3 Perlman, I., Chaikoff, I. L., and Morton, M. E., *J. Biol. Chem.*, 1941, **139**, 433.

† The writer is indebted to the Radiation Laboratory of the University of California, and especially to Dr. J. G. Hamilton, for the supply of radioactive iodine (half life of 8 days) used in this work.

‡ Oxygen consumption tests were very kindly conducted by Mr. Virgil Herring.

follows. The animals were injected subcutaneously 44 hours before autopsy with an aqueous solution (4 cc) of radioactive iodine, as the sodium salt, which had an activity of approximately 2 microcuries per cc. At autopsy, the neck region was exposed, the entire region of trachea and oesophagus bordering the normal site of thyroid tissue was dissected out *in toto* after careful inspection through the binocular dissecting microscope to determine the presence of gross fragments of thyroid which might have been left behind at operation. This tissue was fixed in 10% formalin. A further mass of tissue extending from the base of the tongue to the heart was dissected out and flattened on filter paper in the fresh state, and frozen in the cold room. This mass of tissue included the remaining trachea, hyoid region, base of the tongue, thymus, heart and great vessels, oesophagus, and muscles bordering the trachea. The separate pieces of tissue were mounted upon filter paper, covered with a sheet of thin cellophane, the whole being placed in an X-ray cassette of suitable size. A sensitive "non-screen" X-ray film was placed in direct contact with the cellophane covering the tissues, and the cassette was closed. The cassette with contained materials was kept in the cold room if fresh tissue was being studied. Exposures made over periods of duration of 1½ to 36 hours gave adequate evidence of radioactivity in the whole tissue mounts, not only in preparations in which the thyroid gland was present (normal control), and in grossly incomplete operations, but also in some of the animals which were judged to be completely thyroidectomized on the basis of gross inspection through the binocular microscope. As a further check on the amount of radioactivity present in some of the "incomplete" operations, the activity of certain of the iodine concentrating fragments was determined, employing the Geiger-Müller counter.§ The results are summarized in Table I. Four methods of ascertaining completeness are listed. These are: weight gain after operation, oxygen consumption tests, gross inspection and presence of iodine concentrating tissue as determined by the radioautograph.

Several points are outstanding in the table. Approximately 50% of the animals which showed no visible evidence of thyroid tissue remaining in the neck upon binocular examination gave evidence of the presence of iodine concentrating tissue in the radioautograph. Gross examination was certainly not an adequate test of the completeness of thyroidectomy in this series. Secondly, in this series, it will be noted that a low oxygen consumption is not incompatible with the presence of iodine concentrating tissue in the neck (pre-

§ These determinations were kindly made by Miss Helen Fahl.

TABLE I.
Comparison of Criteria for Determining Completeness of Thyroidectomy.

Thyroidectomized animal, No.	Final body wt, g	Post. body wt gain, g	Gross inspection	Oxygen consumption	Radioautograph	Radioactivity
14	180	32	Complete*	105†	Incomplete*	562‡
15	203	15	"	129	"	1122
16	223	11	"	107	Complete	—
17	186	15	"	102	"	—
18	191	37	"	114	Incomplete	3818
19	192	21	"	131	"	—
20	183	—3	"	129	Complete	—
21	193	7	"	105	"	—
22	162	—3	"	131	Incomplete	—
23	180	35	Incomplete	143	"	2474
24	180	4	Complete	116	"	—
25	207	43	"	114	Complete	—
Normal control						
26	189	48	Normal	169	Normal	200,000

*The term "incomplete" as employed above denotes the presence of thyroid tissue in supposedly completely thyroidectomized animals as determined by gross examination through the binocular microscope, or denotes the presence of iodine concentrating tissue as determined by the radioautograph.

†Oxygen consumption measured by the method of Haldane. Figures represent liters oxygen/m^2/24 hrs.

‡Measurements made on the Geiger-Müller counter in counts/min. Figure for the normal control obtained by electroscope measurement.

sumably thyroid tissue). On the other hand, an animal with an oxygen consumption relatively high (repeated determinations) may show no evidence of iodine concentrating tissue in the entire neck region. This series of animals is too small to draw any definite conclusions as to the relationship between the oxygen consumption and the presence or absence of iodine concentrating tissue after thyroidectomy. This point, however, does merit further attention.

It is of interest that radioautographs of the neck tissues (other than those in the immediate normal location of the thyroid gland) showed no evidence of the presence of aberrant iodine concentrating tissue in this series. That aberrant thyroid tissues may exist is shown by the work of Erdheim[4] for the rat and Kampmeier[5] for the dog and other animals. As has been shown,[2] radioactive iodine may be present in other tissues than the thyroid. Thus in this series, muscle, heart, thymus, trachea and oesophagus all gave positive images on the X-ray film. In no case, however, did the intensity of this image compare with that cast by iodine concentrating tissue in the region of the thyroid gland. The possibility of self-absorption in these tissues was tentatively ruled out by demonstrating positive trans-

[4] Erdheim, J., *Anat. Anz.*, 1906, **29**, 609.

[5] Kampmeier, O. F., *Univ. of Ill. Bull.*, 1937, **35**, No. 12.

mission through a thick mass of tissues of the radiation from a normal thyroid and also from that animal's thyroid region (incomplete thyroidectomy) showing the smallest concentration of radioactive iodine.

The above evidence suggests the possibility of carrying out the same type of search for radioactive iodine in the thyroid employing the electroscope or Geiger counter, using some other tissue as a control for the amount of radioactive iodine present in tissues other than the thyroid gland. Some such procedure for locating aberrant or incompletely removed thyroid tissue is of great importance in any study in which the results depend on showing the complete absence of thyroid tissue in the thyroidectomized animal. It is of interest to note that the sizes of the images cast on the X-ray film by the iodine concentrating tissues are of the same order of magnitude roughly as the determinations on the Geiger-Müller counter. If there is a direct proportion between the amount of radioactive iodine stored in various sized fragments of thyroid gland as compared with the normal, then it would be possible to estimate roughly the amount of gland remaining in the thyroidectomized animal. Thus the fragment in animal No. 14 would be approximately 1/400 the size of the thyroid of the normal control animal.

It has here been assumed that the iodine-concentrating tissue determined by the radioautographic method represents thyroid tissue. Further studies are intended to correlate histological studies with ability to store iodine. The criticism may be made that absence of iodine concentrating tissue does not rule out the possibility of the presence of thyroid tissue. To the best of available knowledge, thyroid is the only tissue able to concentrate iodine in this particular fashion. Further it has not been shown that the thyroid gland or fragments thereof will fail to concentrate such injected radioactive iodine.

Summary. A simple and rapid method is suggested for ascertaining the presence or absence of iodine concentrating tissue (thyroid gland) in thyroidectomized animals. Injected radioactive iodine is assayed by radioautography of the entire neck region, either in the fresh or the fixed state.

13705

Prothrombin Estimation: A Procedure and Clinical Interpretations.

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From the Department of Medicine, New York University, and the Third (New York University) Division, Welfare Hospital, New York City, and the Department of Surgery, Lincoln Hospital, New York City.

This work deals with a standardized modification of estimating prothrombin concentration (or activity). It places especial emphasis upon the difference between the prothrombin times of whole and diluted plasmas, taking into consideration the respective absolute values of these readings. By these 3 measurements it appears possible to detect certain abnormalities of blood not revealed by the prothrombin time of whole or diluted plasma considered alone.

The technic used for the estimation of prothrombin was that described by Link and his coworkers[1] based on the method of Quick, Stanley-Brown, and Bancroft.[2] In the present study two series of estimations were made: one using the thromboplastin-calcium chloride mixture of Link and his students[1] and the other using Russell snake-viper venom*[3,4] in place of rabbit brain extract.

Procedure. 4.5 ml freshly drawn venous blood were added to 0.5 ml M/10 sodium oxalate. Clear plasma was obtained by centrifuging. 0.1 ml whole plasma which had been kept at 37°C was quickly added to 0.2 ml thromboplastin-calcium chloride mixture (also at 37°C) and the time which elapsed before the fibrin clot formed was noted with a stop-watch. The identical procedure was then repeated using 0.1 ml of 12.5% plasma in place of whole plasma.

When venom was substituted for the thromboplastin the technic was as follows:

0.1 ml whole plasma was added to 0.1 ml venom and placed in the constant temperature bath at 37°C for 5-10 minutes. To this was quickly added 0.1 ml M/40 calcium chloride which had been kept

1 Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. B. C.*, 1941, **138**, 1.

2 Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sci.*, 1935, **190**, 501.

* The Russell snake-viper venom was supplied through the kindness of Dr. R. C. Page of Burroughs Wellcome Co.

3 Fullerton, H. W., *Lancet*, 1940, **2**, 195.

4 Page, R. C., and Russell, H. K., *J. Lab. and Clin. Med.*, 1941, **26**, 1366.

TABLE I.

	Thromboplastin-calcium chloride			Russell snake-viper venom		
	Whole plasma	12.5% plasma	Difference	Whole plasma	25% plasma	Difference
Range	20-27	36-47	11-22	12-22.5	20-39.5	7-17.5
Mean	24	41.4	18.5	19.5	29	10.5
Standard deviation	±2.3	±3.8	±3.5	±2.9	±4.6	±2.5

at the same temperature and the time which elapsed before the fibrin clot formed was noted with a stop-watch. The same procedure was repeated with 0.1 ml of 25% plasma in place of whole plasma.

The estimations were done in duplicate.

Dilution of one part of plasma with 7 parts of 0.85% saline permits of a clearly detectable and satisfactorily reproducible end-point when the thromboplastin-calcium chloride mixture is used. With snake venom, this dilution often does not give a reliable end-point, especially when the prothrombin time is prolonged. 25% plasma was found to be better suited for this when venom was used.

The data obtained by the use of these two methods of estimating prothrombin time in two groups of 20 normal individuals is compared in Table I.

The difference between the prothrombin time of whole and of diluted plasma increases as the concentration (or activity) of the prothrombin decreases. This inverse relationship was observed in

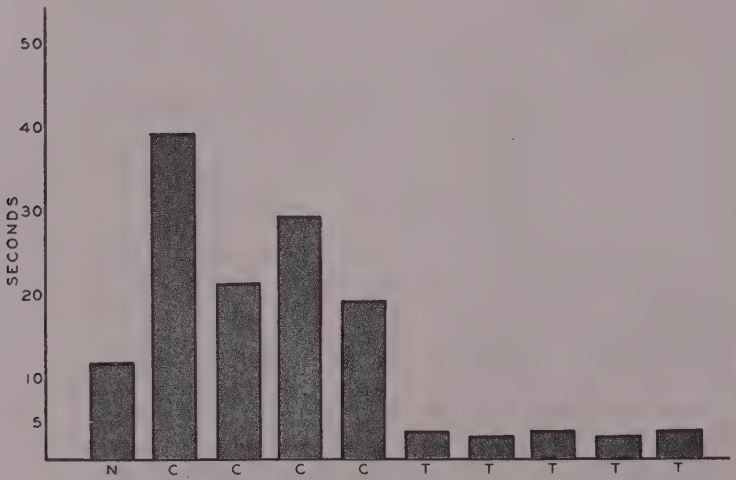

FIG. 1.

The difference between the prothrombin times of whole and dilute plasmas of 10 individuals is shown in stippled columns. N—a normal individual, C—cases of hepatic cirrhosis, T—cases of embolization. Note reduction of difference to below normal in thromboembolization.

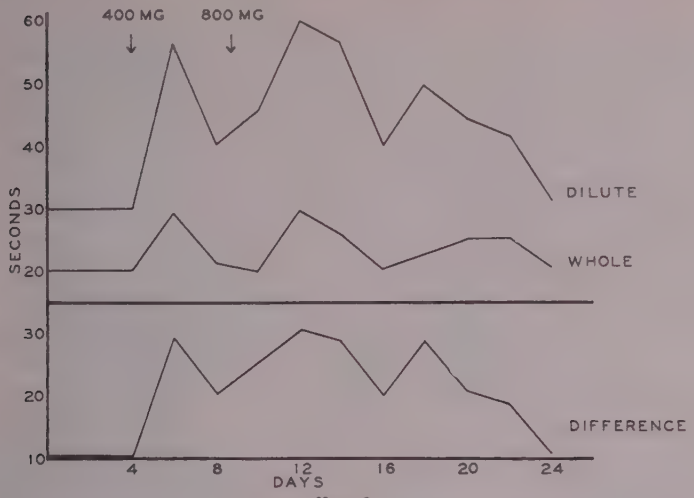

FIG. 2.

The response to different dosages of 3,3′methylenebis (4-hydroxycoumarin) of simultaneously estimated whole and diluted plasma prothrombin times and of the difference is shown. The difference increases considerably more than the whole plasma prothrombin time and is less variable than the dilute.

four cases of cirrhosis of the liver believed to have hypoprothrombinemia (Fig. 1). It is also illustrated in serial estimations following the administration of 3,3′-methylenebis (4-hydroxycoumarin)† which reduces the level (or activity) of prothrombin (Fig. 2). The point at which the dilute plasma prothrombin time becomes substantially prolonged while the whole plasma prothrombin time is only moderately extended becomes an excellent guide to the therapeutic use of this substance.

A state of hyperprothrombinemia is theoretically a possibility. To be able to detect this condition, should it occur, is obviously desirable. If the differences between the prothrombin times of whole and diluted plasma are an expression of the prothrombin concentration (or activity) then it is clear that hyperprothrombinemia is one of the factors responsible for a reduction of this figure to a point lower than that observed in normal plasmas.‡ Such reduction was observed in 5 cases (Fig. 1).

† Commonly designated as "Dicumarol." The compound was supplied through the courtesy of Dr. K. K. Chen of Eli Lilly and Co.

‡ In 2 such cases the difference between whole and 8% plasma was also estimated. In one, the difference (using venom) was 24 sec. while a normal control showed no end point in this dilution. In the second, this difference was 16.8 sec. and that of a normal control 30 sec. (Thromboplastin-calcium chloride mixture used.) This is to be studied further by the method of Warner *et al.*[5]

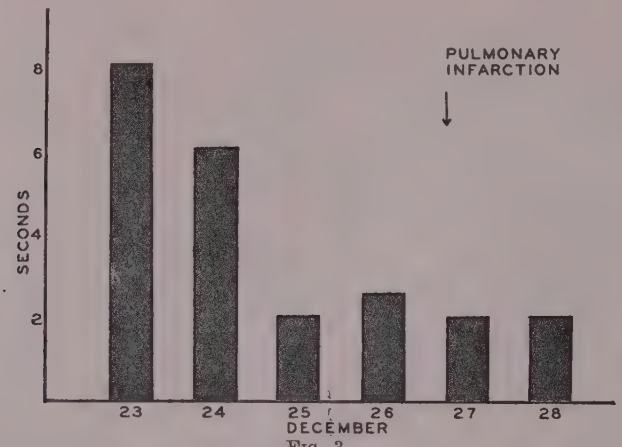

FIG. 3.

Progressive decrease in difference between prothrombin time of whole and diluted plasma (height of stippled columns) in a case of post-operative thromboembolization is illustrated.

In 3 cases of thromboembolization (2 after surgical operations and one postpartum) it was observed that simultaneously with, or one or 2 days prior to, the appearance of signs of pulmonary infarc-

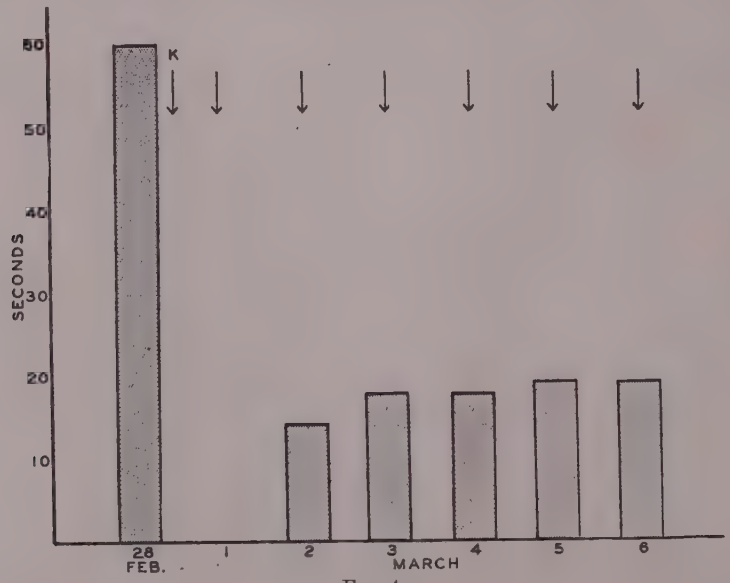

FIG. 4.

The effect of vitamin K in a case of hypoprothrombinemia upon the difference between prothrombin time of whole and 12½% plasma. Therapy was commenced on 2/28/42, after the original estimations. Arrows indicate successive daily injections of 3.2 mg intramuscularly.

tion, the difference between the prothrombin time of whole and diluted plasma became reduced (Fig. 3). The present procedure may, therefore, be of use in selecting patients who are likely to experience thromboembolization (if the observation proves to be constant) and in instituting appropriate prophylactic therapy.

That the extent of the difference between the prothrombin time of whole and diluted plasma is a fairly accurate expression of the prothrombin level (or activity) was further indicated in following the response of hypoprothrombinemic blood to vitamin K therapy (Fig. 4).

The presence of an anticoagulant in the circulating blood has been demonstrated.[6, 7] The possible liberation, under certain pathologic conditions of an excess of this or similar substances into the blood consequently exists. If dilution of these anticoagulant substances renders them ineffectual, it is possible that the prothrombin time of diluted plasma may become as short or even shorter than that of whole plasma at a time when that of whole plasma is lengthened by the activity of the anticoagulant substances. Under these circumstances, the detection of such a condition might be possible by the procedure just described. A case exhibiting a tendency to bleeding suggests that this is so. The prothrombin time of the whole plasma was 51 seconds and that of 12.5% plasma 40 seconds. The estimation of diluted plasma prothrombin time, which would be relatively independent of anticoagulant effect, indicated a normal prothrombin content (or activity), a fact which was verified by the Warner, Brinkhous, Smith method, the result of which was 80% of normal. The reversal of the normal relationship may then be interpreted as excess anticoagulant substances in the blood.

Summary. The difference between prothrombin times of whole and diluted plasma is a more reliable guide to prothrombin activity of the blood than is the prothrombin time of either alone. The difference has been found to be fairly constant in normal individuals. When prolonged, it indicates hypoprothrombinemia. When reduced, it may signify hyperprothrombinemia or an excess of anticoagulants in the blood. The procedure recommended is well suited for following the therapeutic effects of agents which influence the prothrombin level (or activity) such as dicumarol or vitamin K.

[5] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

[6] Howell, W. H., *Am. J. Physiol.*, 1910, **26**, 453; 1911, **29**, 187.

[7] Eagle, H., *J. Gen. Physiol.*, 1934-35, **18**, 531, 547, 818.

13706

Course of Infections with the 3H2 Strain of *Plasmodium cathemerium* in Canaries.

HARRY BECKMAN.*

From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee, Wisc.

The American Society of Parasitologists having given to the strain of *Plasmodium cathemerium* which I have transferred in this laboratory exclusively by mosquito bite since obtaining it from Huff at the University of Chicago, in March, 1938, the designation of "3H2", I wish to record here the course of infections with this strain. For the purpose of the study, 60 uninfected canaries were each bitten by 1 *Culex pipiens* mosquito presumably carrying the sporozoites of this plasmodium, *i. e.*, the mosquitoes had all fed on birds having large numbers of gametocytes of both sexes in the peripheral circulation and, for 10 days or more after the blood meal, had been maintained in a room whose relative humidity is kept at 65% and temperature at 80°F (26.5°C).

The findings may be summarized as follows: (a) of the 60 birds 46 became infected and 44 of these died, thus giving an infection rate of 76.6% and a mortality rate of 95.6%; (b) the incubation period varied from 4 to 11 days but was only 5 to 6 days in 82.6% of instances (in studies not included in the present report I have observed an incubation period of 3 days in one instance and 14 days in another); (c) the duration of fatal infections varied from 1 to 17 days but was from 4 to 9 days in 84.0% of instances; (d) the duration of the active infection in the 2 birds which recovered was 12 and 17 days respectively.

Conclusion. The 3H2 strain of *P. cathemerium* is a very virulent agent when transmitted as indicated in the designation; *i. e.*, exclusively through the bite of infected *C. pipiens* mosquitoes.

* Partially supported by an Eli Lilly and Company grant.

13707

Demonstration of Globi and Leprosy Bacilli by Fluorescence Microscopy.*

HOWARD J. HENDERSON, E. H. SPAULDING AND E. S. GAULT.
(Introduced by E. R. Long.)

From the Henry Phipps Institute, University of Pennsylvania, and the Department of Bacteriology and Immunology, Temple University School of Medicine, Philadelphia.

Hagemann[1] reported the superiority of a fluorescence method using berberine sulfate stain for demonstrating leprosy bacilli in nasal mucus and thick blood smears. The same year he[2] proposed an auramin-fluorescence technic for the routine identification of acid-

A B

FIG. 1.
A. Globi in impression smear from spleen. Approx. 1000 ×.
B. Same field at approx. 400 ×.

* Aided by a grant from the Leonard Wood Memorial for the Eradication of Leprosy (American Leprosy Foundation).

[1] Hagemann, P. K. H., *Deutsche med. Wchnschr.*, 1937, **63**, 514.

fast bacteria, especially tubercle bacilli. The suitability of this method for cultures of *Myco. leprae* has been confirmed by Küster[3] and for leproma smears by Kline and Leach.[4]

Recently one of us (H.J.H.) has investigated the separation and concentration of acid-fast organisms from the tissues of leprosy patients. To cryochemed spleen† distilled water was added and direct impression smears were made from a piece of the tissue. One set of smears was stained with auramin O and examined according to the fluorescence procedure described by Richards and Miller.[5]

A representative field is shown in the accompanying photomicrographs. The faint material in the background is non-acid-fast splenic cellular substance. The acid-fast bacilli have a characteristic granular appearance.

Although no accurate comparison was made with Ziehl-Neelsen stained smears, the fluorescence technic was clearly superior for demonstrating globi and leprosy bacilli. It is of interest to note that Richards, Kline and Leach[6] believed more tubercle bacilli could be demonstrated in the same microscopic fields by fluorescence than by the conventional method.

13708 P

A Method for Separating Intact Leprosy Bacilli from Leprous Tissue.*

HOWARD J. HENDERSON. (Introduced by E. R. Long.)

From the Henry Phipps Institute, University of Pennsylvania.

In a recent investigation the author[1] attempted to obtain from spleens of lepers an antigen or product analogous to tuberculin which might be used in the diagnosis of leprosy. The product, obtained

2 Hagemann, P. K. H., *München. med. Wchnschr.*, 1938, **85**, 1066.

3 Küster, H., *Deutsche med. Wchnschr.*, 1939, **65**, 92.

4 Kline, E. K., and Leach, R. E., *Proc. Conf. State and Prov. Lab. Directors*, 1940.

† Obtained through the courtesy of Dr. John Hanks, Culion, P.I.

5 Richards, O. W., and Miller, D. K., *Am. J. Clin. Path.*, 1941, **11**, 1.

6 Richards, O. W., Kline, E. K., and Leach, R. E., *Am. Rev. Tuberc.*, 1941, **44**, 255.

* Aided by a grant from the Leonard Wood Memorial for the Eradication of Leprosy (American Leprosy Foundation).

1 Henderson, H. J., *Internat. J. Lep.*, 1940, **8**, 271.

by buffer solution extraction of finely ground leprous spleens rich in acid-fast bacilli was tested on leprosy patients by a committee in Manila, P. I.[2] Specific tuberculin-like action could not be demonstrated. There are at least two possible explanations for this negative result. One is that there is no hypersensitiveness to leprosy bacilli. The other is that the acid-fast bacilli in the spleen after the grinding were adsorbed or protected by the preponderantly great amount of normal splenic cells present, and therefore not well extracted.

It seemed preferable, if possible, to separate the bacilli from leprous tissues before grinding and extraction. Previously, attempts have been made to separate acid-fast bacilli from the tissue of the lesions in which they are found. Cowdry[3] and his coworkers dissected bacilli-laden cells from tissues in rat leprosy and then ruptured them with a Mueller press. The expressed tissue fluid, cytoplasm, nucleoplasm and some unruptured cells were ground with sand and centrifuged at low speed. The supernatant fluid, which contained acid-fast bacilli, was then removed and recentrifuged at high speed. The sediment contained a large number of acid-fast organisms. In the experiments here reported advantage was taken of homogenizing chambers[4] for tissue cells and the interface studies of Mudd and Mudd[5] which showed that certain acid-fast organisms, including stock strains of leprosy bacilli, pass readily from the watery into the oil phase.

Leprous splenic tissue was obtained through the courtesy of Dr. John Hanks of the American Leprosy Foundation, Culion, P. I. In order to prevent autolysis in transit the spleens were minced and "cryochemed" to remove practically all the moisture and sealed *in vacuo*.

Sixty-one grams of dehydrated leprous spleens were ground with mortar and pestle, suspended in 0.25% phenolized distilled water, and homogenized at a pressure of 3,000-3,500 lb. Smears of the homogenized material showed that practically all of the splenic cells were disrupted and that very few, if any, of the acid-fast bacteria were injured. The homogenized suspension was then mixed with equal parts of neutral "virgin" olive oil in a shaking machine for one hour. After centrifugation for 15 minutes there were 4 separate distinct layers in the bottles (Fig. 1). Layers 1, 2, and 3 were decanted from layer 4 and allowed to reform in the course of 2 hours

[2] Joint Committee on Leprosy Skin Tests, *Internat. J. Lep.*, 1940, **8**, 263.

[3] Cowdry, E. V., Ravold, A., and Packer, D. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 341.

[4] Chambers, L. A., personal communication.

[5] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1927, **46**, 167.

standing in a separatory funnel. Layer 2 was then separated from the oil (layer 1) and watery (layer 3) layers. Stained smears of all 4 layers were then made, and showed the following: Layer 1, the top layer, was clear olive oil containing no acid-fast bacteria. Layer 2 consisted chiefly of acid-fast bacilli, with a few unruptured tissue cells and some debris. Layer 3 was a cloudy, watery layer and contained an occasional acid-fast bacillus. Layer 4, the bottom layer, consisted of whole tissue cells, debris, and an occasional acid-fast bacillus.

After layer 2 had been separated mechanically from the other layers it was washed several times with acetone and cryochemed. Eight hundred and forty-eight milligrams of dry residue, consisting almost entirely of acid-fast bacilli, were recovered from the 61 g of dried spleen. Studies with these organisms are in progress.

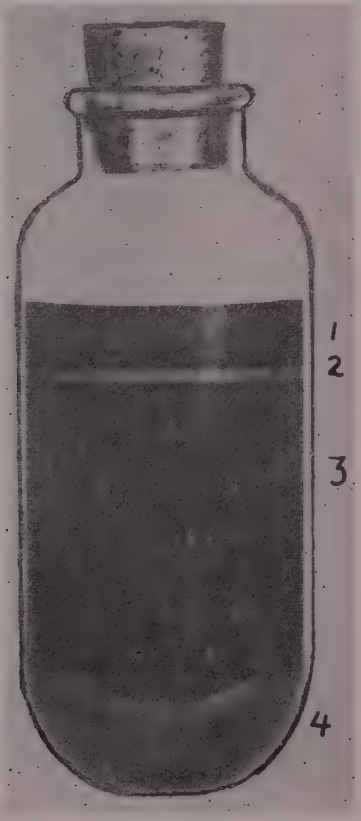

FIG. 1.

13709 P

Effects of Carcinogenic Agents on *Paramecium caudatum.*

Irving A. Tittler and Mildred Kobrin. (Introduced by J. A. Dawson.)

From the Department of Biology, Brooklyn College, Brooklyn, N.Y.

Recently Mottram[1] has shown that when cultures of Paramecia were submitted to long exposures of 3:4 benzpyrene, organisms appeared which were abnormal in size, shape and structure, etc. Such organisms, when isolated and placed in normal culture media, divided and gave rise to populations of abnormal Paramecia. The following account deals with experiments undertaken to learn whether other known carcinogenic agents would produce effects similar to 3:4 benzpyrene on *Paramecium caudatum,* and particularly whether these hydrocarbons would exhibit any differences in the rapidity and potency of their actions, such as Shear[2] and others observed in mice.

The carcinogens employed in these investigations were methylcholanthrene* (0.1% to 1%), Scharlach red (0.1% to 1%) and 3:4 benzpyrene (0.1% to 1%, and more dilute concentrations varying from 1 part carcinogen in 1000 parts of medium to 1 part in 100,000 parts of medium). These were finely suspended in rich hay infusion cultures of *Paramecium caudatum* in Syracuse dishes. Subcultures of both experimental and control animals were made at regular and identical intervals to prevent any contamination on the part of the medium. Similarly, organisms were frequently fixed on coverslips, stained and mounted for cytological study.

Results. In all of the experiments similar abnormalities appeared amongst the treated cultures. These abnormalities took the form of swellings, vacuolizations of the cytoplasm, and particularly blisterings of the pellicle. Monster formations, such as Mottram described, were not seen. Organisms exhibiting these abnormalities, even when isolated in normal media, became sluggish and misshapen, and soon died as noted below.

Differences in the potency and rapidity of action of the carcinogenic agents were indicated by the time of appearance of abnormal-

1 Mottram, J. C., *Nature,* 1940, **145**, 184.

2 Shear, M. J., *Am. J. Cancer,* 1936, **26**, 322.

* The authors wish to thank Professor L. F. Fieser of Harvard University for his generosity in supplying them with methylcholanthrene.

ities, as well as by the relative number of abnormal organisms present. Thus, organisms growing in methylcholanthrene exhibited abnormalities within 30 to 32 days regardless of the different concentrations used. Here only a few organisms were affected. Survival following this treatment averaged 5 to 8 days. Scharlach red yielded almost identical results. However, 3:4 benzpyrene proved to be more effective. Paramecia growing in this hydrocarbon exhibited abnormalities within 12 to 14 days in all of the concentrations employed. These abnormal organisms rarely survived for more than 5 days. Furthermore, the number of organisms affected was considerably greater than either of the other agents employed. Cytological studies of abnormal organisms that were fixed and stained, gave no indication that either the macronucleus or micronucleus were affected or involved.

Conclusion. These results indicate that exposure of *Paramecium caudatum* to the carcinogens employed in these experiments, causes the production of certain abnormalities regardless of the different concentrations of the carcinogenic agents used. The order of potency for these carcinogens, as demonstrated by the time of appearance of abnormalities, is as follows: 3:4 benzpyrene; methylcholanthrene, and Scharlach red.

13710

Cell-Free Enzymes of *Azotobacter vinelandii.**

S. B. LEE, R. H. BURRIS AND P. W. WILSON. (Introduced by E. G. Hastings.)

From the Department of Agricultural Bacteriology, University of Wisconsin, Madison.

Bach and coworkers[1] have reported nitrogen fixation by a cell-free extract of *Azotobacter* cells. Roberg[2] was unable to confirm their results. The possession of a cell-free nitrogen-fixing enzyme system would greatly facilitate studies on the mechanism of nitrogen fixa-

* This work was aided by a grant from the Rockefeller Foundation. We wish to thank Professor J. W. Williams for use of the concentration centrifuge and Dr. Van Potter for a gift of pure cytochrome *c*.

1 Bach, A., Jermoljeva, Z., and Stepanjan, M., *Compt. rend. acad. sci.* (U.S.S.R.), 1934, **1**, 22.

2 Roberg, M., *Jahrb. f. wiss. Bot.*, 1936, **83**, 567.

tion. With this in view, studies reported here describe preliminary investigations on various cell-free enzymes obtainable from *Azotobacter vinelandii*.

Cultures of *A. vinelandii* were grown in Burk's[3] nitrogen-free medium with vigorous aeration for 24 to 30 hours at 30°. Cells were removed from the liquid medium with a Sharples supercentrifuge, washed by resuspending in half-strength Burk's salt solution and again recovered by centrifuging. The washed cell paste was ground† with pH 7.0 phosphate buffer according to the method of Wiggert, Silverman, Utter and Werkman.[4] The glass and larger cell fragments in the ground material were removed by 30 minutes centrifugation at 4500 rpm in an angle head centrifuge. The supernatant was further clarified by 30 minutes' centrifugation at 24,000 rpm in the Beams air driven concentration centrifuge (rotor radius approximately 3 inches). These extracts were diluted with an equal volume of pH 7.0, M 15 phosphate buffer and passed through a Berkefeld N or Mandler 15 filter. Such filtrates showed no growth in a nitrogen-free medium.

Cell-free juices of *Azotobacter* exhibit a powerful hydrogenase. Methylene blue reduction under an atmosphere of hydrogen proceeded at a rate approximately ten times the endogenous reduction rate under helium or vacuum. Methylene blue reduction also revealed hydrogenase activity in toluene preserved cells and in acetone dried powders prepared by the method of Bovarnick.[5]

Gas uptake of cell-free *Azotobacter* juice as measured in the Warburg respirometer in a 95% hydrogen 5% oxygen atmosphere was 540 cmm per hour per mg nitrogen content of the juice, whereas gas uptake under 95% He 5% oxygen was but 15 cmm. This hydrogen-oxygen uptake was inhibited 90% by M 1000 potassium cyanide and 75% by M/100 sodium azide.

The presence of succinic, malic and lactic acid dehydrogenases in cell-free filtered preparations of *Azotobacter* was demonstrated by the methylene blue reduction technic. No activity was evident with glucose, glutamic acid or formic acid as substrate. Sodium succinate oxidation in the Warburg respirometer supported an oxygen

[3] Burk, D., and Lineweaver, H., *J. Bact.*, 1930, **19**, 389.

† Professor Werkman and his colleagues kindly demonstrated the method and certain modifications to the authors. Details of the modified method will be published from Professor Werkman's laboratory.

[4] Wiggert, W. P., Silverman, M., Utter, M. F., and Werkman, C. H., *Iowa State Coll. J. Sci.*, 1940, **14**, 179.

[5] Bovarnick, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 191.

uptake over 6 times the endogenous rate of uptake. Nilsson[6] observed no succinic dehydrogenase activity in vacuum dried or air dried *A. chroococcum* cells, though he obtained methylene blue reduction on hexosediphosphate, ethyl alcohol and glucose with such preparations. The wash water from Nilsson's[6] dry cells contained hexosediphosphate dehydrogenase activity in a cell-free state.

Krampitz and Werkman[7] have recently reported that an oxalacetic acid decarboxylase can be obtained in cell-free juices of *Micrococcus lysodeikticus*. A similar enzyme is present in both cell-free juices and acetone dried cells of *A. vinelandii*. A cell-free preparation released CO_2 from a solution of oxalacetic acid at a rate of 384 cmm per hour per mg nitrogen content of the preparation. This rate was several fold as great as the spontaneous decomposition of oxalacetic acid in the presence of a boiled enzyme preparation. Alpha-ketoglutaric acid was also decarboxylated but at a much slower rate than oxalacetic acid. No activity was evident with pyruvic acid as substrate.

Using the oxidation of ascorbic acid, which spontaneously reduces cytochrome *c*, as a measure of cytochrome oxidase activity, no increase in oxygen uptake was detected when purified heart muscle cytochrome *c* was added to *Azotobacter* juice. This indicates either that the cytochrome oxidase system is not active in the juice studied, or more likely, since cyanide-sensitive oxygen uptake on a succinate substrate could be demonstrated, that heart muscle cytochrome *c* can not act in the cytochrome system of *Azotobacter*. Keilin and Harpley[8] have described a similar lack of activity of heart muscle cytochrome *c* on the cytochrome system of *Escherichia coli*.

Summary. Succinic, lactic and malic dehydrogenases and hydrogenase were demonstrated in cell-free juices of *Azotobacter vinelandii* by the use of the methylene blue reduction technic. Oxygen uptake with sodium succinate as substrate and hydrogen-oxygen uptake with hydrogen as substrate were observed in the Warburg respirometer. Cell-free juices contained oxalacetic acid and alpha-ketoglutaric acid decarboxylases, but not pyruvic acid decarboxylase. Though succinate oxidation was cyanide-sensitive, addition of heart muscle cytochrome *c* did not stimulate the oxidation of ascorbic acid, suggesting that the cytochrome system differs from the animal cytochrome system.

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6 Nilsson, R., *Arch. Mikrobiol.*, 1936, **7**, 598.

7 Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, 1941, **35**, 595.

8 Keilin, D., and Harpley, C. H., *Biochem. J.*, 1941, **35**, 688.

13711 P

Relationship of Pleuropneumonia-like (L) Organisms to Infections of Human Genital Tract.*

LOUIS DIENES AND WILLIAM E. SMITH.

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In a consecutive series of cultures from the genital tract of 129 unselected patients, pleuropneumonia-like (L) organisms were recovered from 23 of 77 cervical cultures, from 1 of 8 vaginal cultures, from 3 of 36 prostatic cultures, and from 1 of 8 cultures of urethral discharges from males.[1]

The 4 male patients who yielded an abundant growth of these organisms all had chronic prostatitis. Gonococci were not demonstrable. One patient had rheumatoid arthritis, one had polyarthritis clinically resembling gonococcal arthritis, one complained of soreness of feet and knees and swelling of fingers. Joint effusions were obtained from the first 2 of these patients, but no organisms could be found in them by smear or culture.

Among the females, considering only those cases in which pleuropneumonia-like organisms were the predominating flora and, excluding cases in which only a few colonies were observed, we find that 5 had chronic cervicitis, 7 had gonorrhea, while in 4 the cervix appeared clean. Among the 9 from whom gonococci were *not* recovered, 1 had rheumatoid arthritis and 3 complained of various skeletal aches and pains. One of these 3 was the wife of the male patient who complained of prostatitis, soreness of feet and knees and swelling of fingers, a finding which suggests the possibility of transfer of the organisms by sexual intercourse.

This mode of transmission is further indicated by another finding, not included in the above series. In this case, a man was admitted to the hospital because of acute tenosynovitis of the left wrist and chronic prostatitis. Pleuropneumonia-like organisms were cultivated from his prostatic secretion. The tenosynovitis cleared up within a few days, and no fluid for culture could be obtained from it. Three

* The expenses of this investigation were defrayed in part by a grant from the Commonwealth Fund. This is publication No. 59 of the Robert W. Lovett Memorial Fund for the study of crippling disease.

1 Smith, W. E., paper presented at meeting of Society of American Bacteriologists, Baltimore, Dec. 30, 1941.

days later, this man's wife entered the hospital because of swelling of both knees of 6 weeks' duration, the onset of her arthritis having occurred 2 weeks after their marriage. The knees were not red or hot and were only moderately painful despite the presence of large effusions. Pleuropneumonia-like organisms were recovered from this patient's cervix, which showed a slight discharge. The right knee yielded 100 cc of synovial fluid which contained 90 mg % sugar and 5,050 white blood cells per cumm, 9% polymorphonuclears, 73% lymphocytes. We were unable to demonstrate any organisms in Giemsa or Gram stained smears of this fluid, and we could not cultivate any organisms from it. This patient was discharged unimproved after one week's hospitalization. Both these patients ran an afebrile course. We are indebted to Dr. O. S. Staples and Dr. H. I. Suby for material from these 2 patients.

In addition to the above patients, we have obtained pleuropneumonia-like organisms from one other case of prostatitis and from more than 100 routine cervical cultures. We are especially indebted to Dr. J. V. Meigs and Dr. C. L. Parsons, who sent us many cervical swabs and specimens from purulent complications. Several of their cases presented the clinical picture of acute gonococcal cervicitis. Cultures and smears were repeatedly negative for gonococci, but pleuropneumonia-like organisms were present in large numbers and in almost pure culture. One patient, an 18-year-old girl, developed an acute vaginitis with purulent discharge 4 days after intercourse. An abundant growth of pleuropneumonia-like organisms were recovered from this discharge. In another case these organisms were recovered in pure culture from a Bartholin gland abscess. In a case of salpingitis, pleuropneumonia-like organisms and a few colonies of diphtheroids were cultivated from the pus expressed from the tubes. In a case of peritonitis secondary to salpingitis, an anerobic bacillus (Genus Bacteroides) was obtained in pure culture from the peritoneal pus, and pleuropneumonia-like organisms were subsequently separated from the cultures of this bacillus as a variant growth form. This patient made a prompt and uneventful recovery from her peritonitis. Gonococci could not be demonstrated in any of these cases.

From these findings, it is apparent that pleuropneumonia-like organisms, like many other bacteria, may live in the female genital tract without causing any obvious pathology; but the evidence strongly suggests that they are capable of acting as pathogenic agents, either alone or in combination with other bacteria, and that they may produce a clinical picture that is similar to gonococcal in-

fection in that a purulent discharge develops and such complications as Bartholin gland abscess, salpingitis and peritonitis may ensue. In males, these organisms have been found thus far only in cases of chronic prostatitis, chiefly in patients with history of previous gonococcal infection. The infection appears to be transmissible by sexual intercourse. Although the strains which we have recovered have bred true through many subcultures, one must bear in mind the possibility that these strains may represent variant forms of bacteria, perhaps of gonococci.[2, 3, 4]

The incidence of arthritis in these patients is of particular interest because arthritis is a common complication of both the natural and the experimental diseases produced in animals by organisms of this group. We have not been able, however, to recover pleuropneumonia-like organisms from diseased joints, although we have examined a considerable number of specimens of synovial fluids, synovial tissues and subcutaneous nodules from arthritic patients.

The methods used for the cultivation and examination of pleuropneumonia-like organisms have been previously described.[5]

13712 P

Effect of Biotin on Reproduction in the Domestic Fowl.

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Numerous studies have shown the importance of biotin in chick nutrition. Hegsted, Oleson, Mills, Elvehjem and Hart[1] described a scaly dermatitis of chicks fed purified diets, which was not cured by pantothenic acid. In further experiments Hegsted, *et al.*,[2] found

2 Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 470.

3 Brown, T. M., *J. Bact.*, 1942, **43**, 82.

4 Dienes, L., *J. Bact.*, in press.

5 Dienes, L., *J. Infect. Dis.*, 1939, **65**, 24.

* Borden & Co. Industrial Fellow.

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

1 Hegsted, D. M., Oleson, J. J., Mills, R. C., Elvehjem, C. A., and Hart, E. B. *J. Nutrition*, 1940, **20**, 599.

2 Hegsted, D. N., Mills, R. C., Briggs, G. M., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1942, **23**, 175.

this type of dermatitis was prevented by biotin concentrates and free crystalline biotin. An increase in growth due to biotin was also reported. Ansbacher and Landy[3] have reported similar results using a heat-treated ration.

In attempting to develop suitable purified rations for the study of the effect of various members of the B complex on egg production and hatchability in poultry we have observed a marked fall in hatchability of eggs produced by hens fed purified rations of low biotin content. In this paper we wish to report studies on this condition, which is prevented by materials rich in biotin and by a biotin-concentrate.

Single Comb White Leghorn pullets were housed in individual laying cages and were artificially inseminated. The birds were started on the experimental diets November 25, 1941. The basal ration (R-4) had the following composition: sucrose 61, casein 18,‡ gelatin 5, salts IV 5,[4] soybean oil 5, solubilized liver extract 4,† fish liver oil 2 (3000 IU vitamin A, 400 A.O.A.C. units D per gm). Crystalline vitamins† were added as follows per kg: riboflavin 5 mg, thiamin 3 mg, calcium pantothenate 15 mg, pyridoxin 8 mg, nicotinic acid 100 mg, and choline 2 g. Crude supplements to the basal ration were added at the expense of sucrose. Groups 1 and 2 received the basal ration for 11 weeks. It was found that eggs produced by hens fed the basal ration would not hatch and biotin supplements were added at this time. Group 1 received 15 μg of a biotin concentrate (S.M.A. No. 5000) per 100 g ration and 3% of kidney residue was added to the ration of group 2. Group 3 was fed the basal ration plus 5% whey concentrate and group 4 the basal plus 3% kidney residue for the entire period. There were 2 birds in group 1, 7 in group 2, 3 in group 3, and 4 in group 4.

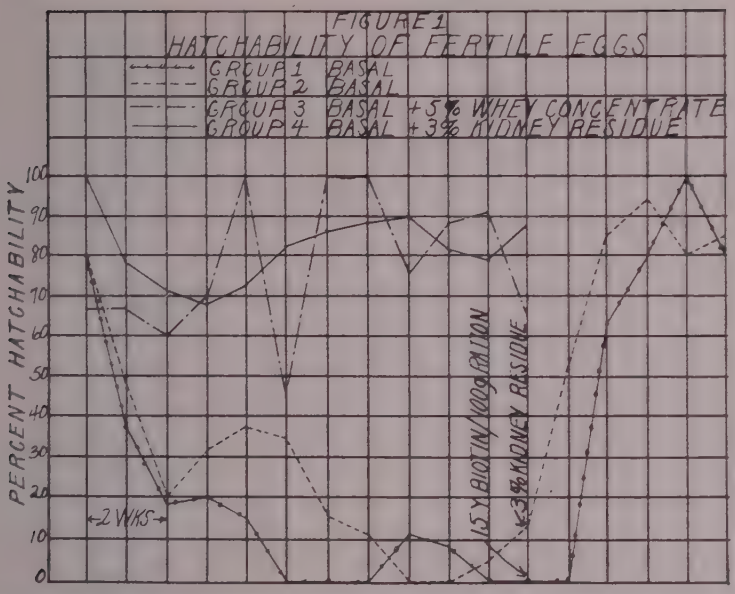
It is apparent from the results in Fig. 1 that the basal ration was inadequate for hens to produce hatchable eggs. A fall in hatchability from approximately 80% of fertile eggs to less than 20% in 6 weeks occurred. That the missing factor was supplied by the whey concentrate or the kidney residue may be seen by comparing the hatch-

[3] Ansbacher, S., and Landy, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 3.

† We are indebted to Borden & Co., Special Products Division, New York, New York, for the fish oil and whey concentrate; to Merck & Co., Rahway, New Jersey, for the crystalline vitamins; and to Wilson Laboratories, Chicago, Ill., for the solubilized liver extract.

[4] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

‡ Borden's H5P. Unobtainable during present emergency.



ability of eggs from groups 3 or 4 with that of the hens fed the basal ration. The results for groups 3 and 4 are not presented beyond the 11th week because the supplements had proven effective by that time and thus were withdrawn so as to deplete these birds for further study.

The addition of kidney residue or the biotin concentrate to hens in groups 1 and 2 respectively brought about a rapid increase in hatchability of eggs produced by these birds. In 3 weeks the percent of hatchability had risen from approximately 10% to at least 80% in both groups.

It might be questioned whether factors other than biotin are involved but this seems unlikely inasmuch as Hegsted, *et al.*,[2] have shown that biotin concentrate and free crystalline biotin replaced kidney residue in chick rations similar to that used in these studies. The cost of the crystalline biotin prohibited its being used in these studies. Whether biotin is the heat labile factor reported by Bauernfeind and Norris[5] to be necessary for avian reproduction cannot be determined from the present study.

The egg production data are not presented inasmuch as there seemed to be little effect of this deficiency on egg production. It is possible that sufficient biotin was present in the basal ration to sup-

[5] Bauernfeind, J. C., and Norris, L. C., *J. Nutrition*, 1939, **18**, 579.

port adequate egg production. No dermatitis was observed during the experiment.

Summary. Evidence is presented to show that biotin is essential for normal embryonic development in the hen's egg. A satisfactory synthetic ration for use in studies of the vitamin requirements of laying and breeding hens is also presented.

13713

Studies on Inhibition and Activation of Atropinesterase.*

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In an earlier communication dealing with the enzymatic hydrolysis of atropine by rabbit serum a study was made of the kinetics of the reaction, its enzyme-substrate affinity relations, and effects of pH and temperature.[1] Subsequently the occurrence of atropinesterase in rabbits, its distribution in blood and organs, and certain of its specificity characteristics were reported.[2] The present study extends these observations to include the influence on activity of certain salts, dyes, sulfhydryl compounds, substances affecting sulfhydryl groups, and physostigmine.

Rabbit serum, used as a source of enzyme, was dialyzed in 10 ml portions against distilled water for 3 days at 5-10° in cellophane tubes. After dialysis the volume was about 15 ml and the serum was centrifuged to remove precipitated globulins. Each batch, kept in a refrigerator, was used within a week. A loss in activity could be observed when the material was used after the first week.

The enzyme activity was determined at 30° and pH 7.4 by the manometric method employing the Warburg apparatus[1, 2] with the substitution of 0.20% $NaHCO_3$ for bicarbonate-Ringer solution in order to eliminate possible effects of KCl, NaCl, and $CaCl_2$. Three ml of substrate solution, containing 10 mg atropine sulfate dissolved in the $NaHCO_3$ solution, were placed in the main chamber; 0.5 ml of the dialyzed serum preparation and 0.5 ml of a solution of the compound to be tested, dissolved in $NaHCO_3$ solution, were placed in the side

* Aided by a grant from the Sidney C. Keller Research Fund.

1 Glick, D., *J. Biol. Chem.*, 1940, **134**, 617.

2 Glick, D., and Glaubach, S., *J. Gen. Physiol.*, 1941, **25**, 197.

arm. Saturation with 95% N_2-5% CO_2 was carried out as usual. In parallel control experiments 0.5 ml of distilled water was used instead of 0.5 ml of enzyme solution, and corrections for non-enzymatic hydrolysis were applied.

The compounds, whose effects on atropinesterase were studied, were obtainable in pure form with the exception of Congo red and methylene blue.† The Congo red was purified by 3 recrystallizations from 70% alcohol, and the methylene blue by 3 recrystallizations from 95% alcohol and washing with petroleum ether.

In the experiments dealing with the effect of calcium ion, atropine could not be used in the form of its sulfate due to precipitation of calcium sulfate; hence the free base of the alkaloid was employed, and HCl was added, after the $CaCl_2$, to bring the pH to 7.4.

From Fig. 1, it is apparent that equimolar concentrations of KCl, NaCl, NaBr, or NaI produce equivalent activations of the enzyme. The concentration-activation curve is very much like that obtained for the effects of KCl and NaCl on cholinesterase in dialyzed rabbit serum.[3] In both cases, with increasing concentration, the curves approach 50% activation in an asymptotic manner. It might be sup-

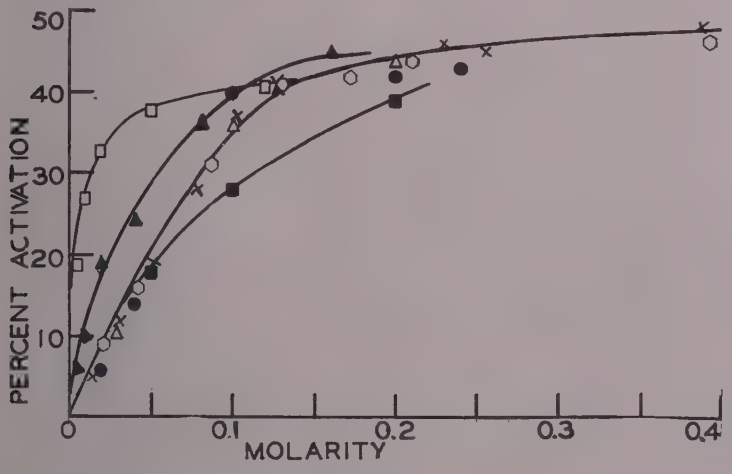

FIG. 1.
Activation of Atropinesterase by Salts.
(○) NaCl, (×) KCl, (●) NaBr, (△) NaI, (▲) Na_2SO_4, (□) $MgSO_4$, (■) KCNS.

† p-Chloromercuribenzoic acid, o-iodosobenzoic acid, and phenylmercurihydroxide were graciously supplied by Dr. Leslie Hellerman of the Department of Physiological Chemistry, Johns Hopkins School of Medicine.

[3] Glick, D., *Nature*, 1941, **148**, 662.

posed that these activations result from effects of the ions on the colloidal enzyme protein. The greater activations produced by equimolar concentrations of the salts with divalent radicals, Na_2SO_4 and $MgSO_4$, are compatible with this view, although Mg^{++} is known to activate certain esterases more specifically.

From Table I it may be seen that the sulfhydryl compounds, cysteine and glutathione, inhibit the enzyme, whereas the effect is reversed with cystine. Since agents capable of eliminating sulfhydryl groups, such as cyanamide,[4] p-chloromercuribenzoic acid,[5] o-iodosobenzoic acid,[6] phenylmercurihydroxide,[5] had no effect, it follows that the dialyzed serum contained no sulfhydryl groups capable of affecting the enzyme activity, nor were sulfhydryl radicals an essential part of the enzyme itself. How much of the cyanide inhibition may be due to reduction of -S-S-groups possibly present in the enzyme preparation cannot be stated.

The inhibition produced by KCN was noted earlier by Van der Heyde[7] although no really quantitative study was made. Bernheim and Bernheim[8] reported an 80% inhibition of the hydrolysis of homatropine by guinea pig liver in the presence of 1.5% (0.36M) NaF, as well as strong inhibitions by physostigmine in concentrations of 0.7×10^{-5} and 2.1×10^{-5} M. The time course of the reactions inhibited by physostigmine in the present investigation is given in Fig. 2. It may be seen that the inhibitions produced by the lower

TABLE I.
The Effect of Certain Compounds on the Activity of Atropinesterase.

Compound	Range of concentration (molarity)	% change
Calcium chloride	.010	15 activation
Trisodium citrate	.030-0.170	0
Sodium cyanide	.005-0.015	10-58 inhibition
Sodium fluoride	.005-0.100	6-59 "
Cysteine	.050-0.200	13-75 "
Glutathione	.008-0.040	17-100 "
Cystine	.005-0.010	10-24 activation
Cyanamide	.006-0.300	0
p-chloromercuribenzoic acid	.25% suspension	0
o-iodosobenzoic acid	"	0
Phenylmercurihydroxide	"	0
Congo red	.00018	0
Methylene blue	.00033	0
Physostigmine salicylate	.0000024-0.00242	36-100 inhibition

[4] Glaubach, S., *Arch. Exp. Path. Pharm.*, 1926, **117**, 247, 257.

[5] Gemmill, C. L., and Hellerman, L., *Am. J. Physiol.*, 1937, **120**, 522.

[6] Hellerman, L., Chinard, F. P., and Ramsdell, P. A., *J. Am. Chem. Soc.*, 1941, **63**, 2551.

[7] Van der Heyde, H. C., *Arch. neerl. Physiol.*, 1921, **5**, 380.

[8] Bernheim, F., and Bernheim, M. L. C., *J. Pharm. Exp. Therap.*, 1938, **64**, 209.

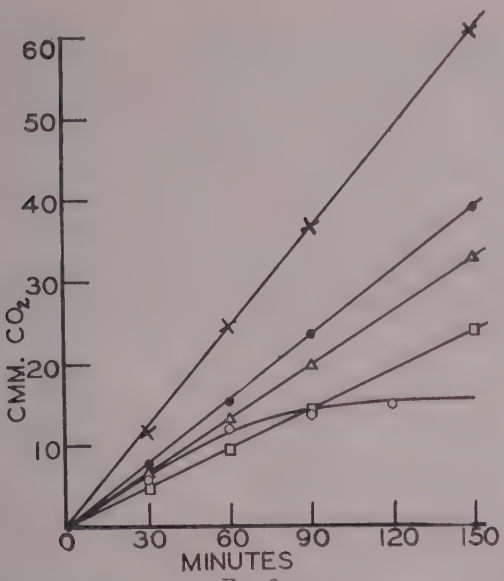

FIG. 2.

Inhibition of Atropinesterase by Physostigmine.
(×) control without drug, (●) 0.24×10^{-5} M, (△) 2.42×10^{-5} M, (□) 24.2×10^{-5} M, (○) $242. \times 10^{-5}$ M physostigmine salicylate.

concentrations of physostigmine reach their maximum during the period of setting up the experiment (about 30 min.) since the hydrolysis-time curves are linear. But, with the highest concentration of inhibitor (242×10^{-5} M), the maximum inhibition is not attained for about 120 min. The lag in obtaining inhibitions of cholinesterase with this drug is well known.

Massart and Dufait[9] found Congo red to be representative of certain acid dyes having no effect on cholinesterase, whereas methylene blue, containing a quaternary ammonium base, strongly inhibited the enzyme in very high dilutions. Rentz[10] had previously shown that methylene blue inhibited cholinesterase as powerfully as physostigmine. Although the concentration of methylene blue used in this case completely inhibited the cholinesterase in the serum, it had no influence on the atropinesterase. This finding affords another mark of differentiation between the two azolesterases.

Summary. Atropinesterase of rabbit serum was found to be activated to the same degree by equimolar concentrations of KCl, NaCl, NaBr, and NaI. Activations were also produced by KCNS,

[9] Massart, L., and Dufait, R. P., *Enzymologia*, 1941, **9**, 364.
[10] Rentz, E., *Arch. exp. Path. Pharm.*, 1940, **196**, 148.

$MgSO_4$, and $CaCl_2$. Trisodium citrate had no effect. Cyanide, fluoride, physostigmine and sulfhydryl compounds inhibited the enzyme. Compounds capable of eliminating sulfhydryl groups present in the enzyme preparation had no influence, nor did Congo red or methylene blue.

13714

Inactivation of Pressor Amines by Quinones and Related Diketones.

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In the metabolism of certain aromatic l-amino acids in the kidney pressor amines are formed by decarboxylases as intermediary products. In the normal kidney the enzyme amine oxidase deaminizes these amines to inert substances. This oxidase is not effective in the ischemic kidney, and the process is arrested at the stage of the pressor amine. This mechanism is thought to be one of the causes of experimental hypertension (Holtz,[1] Bing[2]).

It was believed that an approach to the problem of hypertension could be effected by causing the oxidative destruction of pressor amines circulating in the blood stream. Kisch[3] has shown that certain quinones act as catalysts in the oxidative deamination of amino acids and polypeptides.

Since catechol derivatives and aliphatic enediols form quinones and diketones respectively on aeration in the presence of certain metallic ions as catalysts, it was of interest to examine their effect on various pressor amines *in vitro*. The quinones (or diketones) formed could act either by deaminating the amines or by forming compounds of the azophenine type.

Experimental. Solutions containing tyramine, indolethylamine,

*Hernsheim Fellow in Chemistry.

† George Blumenthal, Jr., Fellow.

1 Holtz, P., Heise, C., and Luedtke, C., *Arch. exp. Path. u. Pharm.*, 1938, **191**, 87.

2 Bing, R. J., *Am. J. Physiol.*, 1941, **132**, 497.

3 Kisch, B., *Oppenheimer's Handb. der Biochem.*, Jena, 1933, Ergw. **1**, 563.

4 Beyer, K. H., *J. Pharm. Exp. Ther.*, 1941, **71**, 394.

ephedrine, benzedrine and paredrine were aerated in the presence of catechol, dihydroxyphenyl-alanine (dopa), reductone, gallic acid, *l*-ascorbic acid, *d*-glucoascorbic acid, and *p*-benzoquinone. Copper, cobalt, nickel and iron salts were used as catalysts. At the same time blanks composed of the pressor amines and the metal catalysts but without the quinone precursor or the aliphatic enediols were similarly aerated.

After aeration the solutions were filtered and 1 cc was injected intravenously into nembutalized cats whose femoral artery was cannulated for blood pressure measurements. The controls gave the immediate response typical of the pressor amine used, but aeration

TABLE I.

Destruction of Pressor Amines During Autoxidation of Quinone Precursors and by Quinones.

Quinone precursors	Bivalent metal ion	Pressor substance used: Tyramine or Indol-ethylamine	Ephedrine	Benzedrine or Paredrine	Renin*
		% destruction of pressor amines inferred by diminished response			
None		0	0	0	S
d,l-Dopa	Cu, Co or Ni	100	100	30	100
d,l-Dopa	Fe	80	0		
Catechol	Cu, Co or Ni	100		20	100
Catechol	Fe	80			
Gallic Acid	Cu	20			
l-Ascorbic Acid†	Cu	30			
d-Glucoascorbic Acid‡	Cu	30	30		
Reductone†	Cu	100		30	
Quinones					
p-Benzoquinone					
Non-aerated		30	70		
Aerated		100	20	100	
"Dopa" Quinone					
Non-aerated		20			0
Aerated		60			100

S = slight.
* = See legend below.
† = 20 mg per cc of incubated mixture.
‡ = 40 mg per cc of incubated mixture.

A solution was made up from 2 cc of a 0.5% solution of quinone precursor, 2 cc of a 0.05% solution of pressor amines and 1 cc of a 0.02% solution of the metal catalysts. (In case of *l*-ascorbic acid and reductone of 5% solution and in case of *d*-glucoascorbic acid a 10% solution was taken.) These solutions were aerated at room temperature for about 16 hours. 1 cc of each solution, thus containing 0.5 mg of pressor amine and 0.2 mg of the sulfate of the respective metal ion plus 2 mg of quinone precursor, was injected intravenously into nembutalized cats. In the case of renin 3.0 cc of a bicarbonate extract of acetone dried hog kidney were aerated for 3 hours with the oxidation catalysts and quinone precursors. 1 cc containing 0.5 cc of the original extract was injected. The results given represent the average value of single observations on 3 different animals.

of the solutions in the presence of dihydroxyphenyl-alanine, catechol or reductone had completely destroyed the pressor effect of tyramine and indolethylamine. Aeration in the presence of gallic, *l*-ascorbic or *d*-gluco-ascorbic acids caused partial destruction of the pressor effect of these amines. Renin prepared from acetone-dried hog kidney was likewise inactivated by the quinone precursors and other diketones tried. Ephedrine, benzedrine and paredrine were only partially destroyed in most cases (Table I).

It is interesting to note that when tyramine or indolethylamine were added to *p*-quinone, only partial destruction of the amine took place if the solution was not aerated, whereas total destruction took place during aeration. A similar effect was noticed with "dopa" quinone obtained from dihydroxyphenyl-alanine by oxidation with potassium ferricyanide at pH 8.

The same system may destroy the pressor substances produced in the ischemic kidney. The daily injection of a mixture of copper sulfate and dihydroxyphenyl-alanine into hypertensive rats, made hypertensive by perinephric scar and observed for over 3 months, gave significant drops in blood pressure, averaging about 34 mm mercury.[5]

The same amounts of copper sulfate or dihydroxyphenyl-alanine, each injected alone, had no effect on the blood pressure of the hypertensive animals. The same mixture, as above, injected during a 6-day period into normal rats did not produce any change of blood pressure. No toxic effects were observed after any of these injections (Table II).

Discussion. Schroeder[6] has described the reduction of blood pressure in hypertensive animals by the injection of mushroom tyrosinase. The above observations may indicate that the suggested oxidative destruction of pressor amines by tyrosinase is achieved through the intermediate oxidation of catechol derivatives such as dopa and epinephrine, resulting in the formation of ortho-quinones as hydrogen acceptors. Bing[7] has observed that the pressor activity of dihydroxyphenylethylamine is destroyed by oxygen in the presence of kidney extract, whereas tyramine is not so destroyed. This effect can also be explained by the intermediate formation of an ortho-quinone.

[5] A drop in blood pressure of 24 mm mercury as measured by arterial puncture was also obtained in a "Goldblatt" dog by intramuscular injection on two successive days of 300 mg "dopa" and 30 mg copper sulfate.

[6] Schroeder, H. A., and Adams, M. H., *J. Exp. Med.*, 1941, **73**, 531.

[7] Bing, R., Zucker, M. B., and Perkins, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 372.

TABLE II.
Blood Pressure Responses of a Group of Hypertensive and Normal Rats to injection of Metal Catalysts and Quinone Precursors.

Group	No. of rats in group	Treatment	Avg blood pressure for group, successive days								
			1	2	3	4	5	6	7	8	9
	Hypertensive Rats										
A	4	0	226	224	220	216	218	226			
B	2	1 mg $CuSO_4$	206	214	206	212	208	212			
C	2	10 mg Dopa	238	232	230	238	242	228			
D	6	1 mg $FeSO_4$ + 10 mg Dopa	194	190	186	186	182	*	(178)		
D	6	Same rats; 1 mg $CuSO_4$ + 10 mg Dopa	178	172	168	166	160	*	(164)		
D	6	Same rats without treatment	164	166	170	168	170	172	189	—	190
	Normal Rats										
E	4	1 mg $CuSO_4$ + 10 mg Dopa	128	134	134	134	136	136	130	132	

*No injection this day.

Blood pressure was measured in the rats' tails according to Williams, Harrison, and Grollman.[5] Each rat in group B received 1 cc of a 0.1% $CuSO_4$ solution, each in group C 1 cc of a 1% dopa solution by daily intramuscular injection for a 6-day period. The 6 rats of group D and E received a course of daily intramuscular injections of 1 cc per day and animal of a solution containing 1 mg of the metal sulfate and 10 mg of dopa.

Summary. 1. The pressor activity of certain aromatic amines is destroyed by aeration *in vitro* in the presence of quinones. 2. Simultaneous injection of dihydroxyphenyl-alanine and small amounts of copper salts in hypertensive rats causes a considerable lowering of blood pressure. 3. It is suggested that tyrosinase reduces the blood pressure by its oxidative effect on acceptor substances of the catechol type such as dopa and epinephrine, which in turn, upon oxidation to ortho-quinones, destroy the pressor amines *in vivo*.

We are indebted to Dr. Harry Sobotka for his friendly advice and criticism throughout the course of this work.

We wish to thank Messrs. Smith, Kline and French for samples of benzedrine and paredrine.

[5] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

13715

Differential Characters of Two Strains of *Clostridium botulinum*, Type E: Action of Toxins in Chickens.

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The isolation and identification of 2 strains of *Clostridium botulinum*, type E, incitants of 2 fatal cases of human botulism in New York State, have been reported.[1, 2, 3] Since type E had not then been demonstrated as an incitant of human botulism in this country, a differential study of the strains was undertaken, the results of which are herein recorded. A third outbreak with which a type E strain was associated has recently been reported by Geiger.[4]

One strain of *Cl. botulinum* (No. 35396) was isolated from German-canned sprats;[1] the other strain (No. 36208) was recovered from smoked salmon that had come from Nova Scotia.[3]

Colony Formation. On 5% defibrinated horse blood agar plates[5] the colonies of the "sprat" strain vary in shape and size, have a comparatively granular surface, a slightly raised center, and a narrow, flattened, irregular periphery. The colonies of the "salmon" strain are more regularly round, have a smoother surface, and a narrow, flattened, usually entire, periphery.

Agglutinative Properties. Both strains are agglutinated to a significant degree in homologous antiserum only and the agglutinative properties are absorbed from these antisera by the homologous culture only, indicating the sprat and salmon strains to be of distinct agglutinative groups.

Toxigenic Activity. Guinea pigs, mice, rabbits, and kittens were all highly susceptible to both sprat and salmon toxins injected subcutaneously. Chickens also were highly susceptible to the salmon toxin but not to the sprat toxin. Since it had been found that the

1 Hazen, E. L., *J. Infect. Dis.*, 1937, **60**, 260.

2 Hazen, E. L., *Science*, 1938, **87**, 413.

3 Mackenzie, G. M., Three cases of botulism treated with antitoxin, *Clinical Miscellany, Mary Imogene Bassett Hospital, Cooperstown, New York*, Springfield, Ill., Thomas, 1934, Vol. I, p. 53.

4 Geiger, J. C., *J. Am. Med. Assn.*, 1941, **117**, 22.

5 Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 2d ed., Baltimore, Williams and Wilkins Company, 1939, p. 104.

antitoxins produced against each toxin neutralized the toxins reciprocally, a further study was undertaken upon this evidence in chickens of a difference between the toxins.

White Leghorn chickens from 4 to 12 weeks old and weighing from 210 to 525 g were employed. The toxic filtrates were obtained from cultures grown in portions of the same lot of broth medium, under similar conditions. Twenty-nine chickens were tested for susceptibility to subcutaneous doses of the fluid toxins, 9 with doses ranging from approximately 300 to 4000 guinea pig M.L.D. of the sprat toxin and 20 with from approximately 4 to 300 guinea pig M.L.D. of the salmon toxin. None of the chickens that received the sprat toxin became ill, while all that had the salmon toxin showed symptoms characteristic of botulism and many died. The fatal dose for these chickens, which weighed from 230 to 315 g, was approximately 6 to 8 guinea pig M.L.D. (It is interesting that the toxin of the German sprat strain and that of a Russian strain isolated from fish and identified as type E by Gunnison, Cummings, and Meyer,[6] and transfer of which Doctor Meyer kindly supplied, acted similarly in chickens; the agglutinative properties of the two strains were also found to be similar.)

Attempt was then made to determine whether the blood of normal chickens contained a neutralizing substance for the sprat, but not for the salmon toxin. Toxin-antitoxin neutralization tests were performed in mice with (1) mixtures of sprat or salmon toxin and normal chicken serum; (2) mixtures of sprat or salmon toxin and blood of chickens immunized respectively against each toxin. (Toxoid was employed for immunization against the salmon toxin.) Briefly, from 0.5 to 1 ml of normal chicken serum afforded mice no protection against less than 2 mouse M.L.D. of either toxin, whereas 0.25 ml of sera from the immunized chickens protected mice reciprocally against from 4 to 8 M.L.D of the salmon and sprat toxins.

Since a neutralizing substance was not demonstrated in normal chicken serum, a further possibility was investigated: whether the sprat toxin was destroyed after absorption from the subcutaneous tissue; or, if not destroyed, for how long a time after injection it could be detected in the circulating blood and excreta.

Four normal chickens were bled from the veins on the ventral surface of the wings, and were then given subcutaneous injections of from approximately 2100 to 4000 guinea pig M.L.D. of the toxin. Blood was collected from 3 in one hour, from the fourth in 2 hours,

[6] Gunnison, J. B., Cummings, J. R., and Meyer, K. F., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 278.

and from all 4 at 24-hour intervals for 96 hours. From 0.5 to 1 ml of the whole blood was injected immediately into mice, intraperitoneally. None of those injected with normal blood became ill; all injected with blood obtained from the chickens up to 48 hours after injection of the toxin died with symptoms of botulism. Two of 4 mice injected with 72-hour specimens died. None that received 96-hour specimens were ill.

Fifteen specimens of excreta were collected at intervals of from one hour to 24 hours for the 96 hours, emulsified in physiological salt solution, and refrigerated overnight. The material was then centrifuged at high speed and from 1 to 1.5 ml of the supernatant was injected intraperitoneally into mice. Only 3 of the 15 specimens gave rise to botulism; they were from 3 different chickens and had been collected 24, 48, and 72 hours after injection of the toxin.

Summary. Differential characters of two strains of *Clostridium botulinum*, type E, are described. One strain was isolated from German-canned sprats; the other from Nova Scotian smoked salmon. Culturally they can be differentiated by colony formation on horse blood agar plates. Agglutination and absorption tests indicate them to be of distinct serologic groups.

The toxins of both strains were neutralized reciprocally by their antitoxins. Tests for toxigenic activity demonstrated, however, that the toxins were not identical. Subcutaneous injections of both toxins uniformly gave rise to symptoms characteristic of botulism in guinea pigs, mice, rabbits, and kittens. White Leghorn chickens were highly susceptible to the toxin of the salmon strain but not to that of the sprat strain. In chickens 4 to 12 weeks old, approximately 6 to 8 guinea pig M.L.D. of the salmon toxin gave rise to fatal botulism, while doses of the sprat toxin several hundred times as large failed to kill or induce symptoms of botulism. The sprat toxin was demonstrable in the blood and also in the excreta of some of the chickens 72 hours after inoculation.

13716 P

Specific Substances in the Urine of Leucemia Patients.

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In previous reports[1, 2] we have shown that extracts of the urine of patients with leucemia contained substances capable of inciting cellular proliferation in the organs of guinea pigs similar to those found in human leucemias. In one of these reports[2] it was shown that the substances obtained were specific for the type of leucemia from which the urinary extracts were made.

It has seemed obvious that the chloroform extracts described in the earlier communication contained many inert materials as well as the active principles. Because of this we decided to fractionate the chloroform extracts and tried to trace the active principles preparatory to chemical analysis.

The following method has been developed to fractionate the extracts of urine of patients with chronic myeloid and chronic lymphoid leucemia. It applies particularly to the fractionation of the former.

The urine is extracted with chloroform as before.[2] The chloroform is removed by distillation and the residue is suspended in 200 to 300 cc of 10% sodium hydroxide. This suspension is extracted 8 to 10 times with 100 cc portions of ethyl ether to remove any substances which will not react with sodium hydroxide. The fraction soluble in ethyl ether (A) is recovered by removal of the solvent. The residual alkaline solution (B) is reacidified with 30 to 50 cc of concentrated hydrochloric acid. The acid solution is extracted with chloroform, the extract after removal of the chloroform is extracted with successive portions of petroleum ether (C); these extracts are combined, distilled *in vacuo*. The residue crystallizes readily in the cold.

Various quantities of urine have been handled in this manner; 30 to 50 liters yield from 3 to 6 g of the petroleum ether-soluble fraction.

The 3 fractions of the extract from the urine of patients with chronic myeloid leucemia have been injected into guinea pigs. Four animals given the alkali-soluble fraction (B) exhibited the same

1 Miller, F. R., Wearn, J. T., and Heinle, R. W., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 479.

2 Miller, F. R., and Hause, W. A., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 387.

changes that we have reported previously. Three animals received the ethyl ether-soluble fraction (A) and although they became ill they showed no proliferation of myeloid cells. Five guinea pigs were given the petroleum ether-soluble fraction (C) in 3 or 4 doses of ½ cc each (50 to 140 mg per dose in olive oil) on successive days and the animals were observed until they showed signs of being ill. Autopsies were performed 3 to 4 weeks after the first injection and proliferation of myeloid cells in the liver, lungs, spleen and adrenals was greater in extent than in any of our previous animals. Three of these animals had white blood cell counts of 40,000 the day of autopsy. The blood smears contained 10 to 20% myelocytes, a few myeloblasts and low percentages of lymphocytes.

Proliferation of lymphoid cells occurred after the use of each of the 3 similar fractions of the extracts obtained from the urine of patients with chronic lymphoid leucemia with no apparent variation. This method, therefore, does not aid in tracing the active principle of this extract.

For control experiments 30 liters of urine of normal individuals were fractionated in the same manner. The yield of the final petroleum ether-soluble fraction was less than one gram. This material produced no specific cellular response.

A method is presented for partially purifying the active principle found in chloroform extracts from the urine of patients with chronic myeloid leucemia. This method does not apply to similar extracts from the urine of patients with chronic lymphoid leucemia.

13717

Percutaneous Application of Sulfanilamide in Animals and Men.

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As has been described,[1] it is possible to obtain a chemotherapeutic effect in animals and men by the percutaneous application of external disinfectants. In this respect special attention was given to p-chloro-

[1] Zondek, B., in press.

xylenol and to some of its derivatives. By the percutaneous application of p-chloro-xylenol in the form of tinctures or concentrated ointments (30%) it was possible to save rats from deadly pneumococci infections. Staphylococci inoculated in the blood of patients treated with p-chloro-xylenol percutaneously were inhibited in their growth. Furthermore, the percutaneous applictaion of p-chloro-xylenol brings about a disinfection of the urinary tract following the excretion of the drug with the urine. Clinical studies in cases of urogenic and general infections have confirmed the value of this percutaneous chemotherapy.

The results obtained with this method of treatment have encouraged us to study also the cutaneous resorption of sulfanilamide. These studies have shown that sulfanilamide also is absorbed by the skin and is present in the blood following its cutaneous application.

Method. Animal experiments were performed in rabbits. Sulfanilamide was given in organic solvents, since the percutaneous application of ointments yielded less satisfactory results. Our experience with the percutaneous use of estrone[2] has shown the advantages of organic solvents for cutaneous absorption. Sulfanilamide was applied to the shaved skin of the back, out of reach of the animal's tongue, to eliminate a possible oral absorption. Each animal was then kept in a separate cage.

The solutions of sulfanilamide for percutaneous use: For experiment No. 1, with rats, a 2.5% tincture in alcohol was used. Later we found that the percutaneous absorption of sulfanilamide is augmented by the addition of glycerine and soap. Hence the solutions for experiments Nos. 2 and 3 were prepared as follows: 15 g sulfanilamide were dissolved in 75 cc of acetone, 15 cc glycerine, 2.5 cc liquid soap and 7.5 cc water. For subcutaneous or intramuscular use we used soluseptazine (phenyl-propyl-amino-benzene-sulfamide-α-γ-disulfonate sodium).

Experimental. The first series of experiments aimed at evaluating the concentration of the drug in the blood of rabbits, thus enabling a comparison of the concentrations obtained by other routes of administration. The second series of studies was conducted in men. Sulfanilamide estimations in the blood were carried out according to Werner.[3]

(1) Experiments in rabbits: Five rabbits weighing 1500 g each received 0.5 g of sulfanilamide. This was administered in 3 animals percutaneously, in one orally, and in one subcutaneously. For inunc-

[2] Zondek, B., *Lancet*, 1938, **234**, 1107.

[3] Werner, M., *Lancet*, 1939, **236**, 18.

TABLE I.
Sulfanilamide Blood Concentration in Rabbits after Percutaneous, Peroral and Subcutaneous Administration of 0.5 g of Sulfanilamide.

Hr after treatment	R I Received 0.5 g sulfanilamide percutaneously mg%	R II 0.5 g sulfanilamide percutaneously mg%	R III 0.5 g sulfanilamide percutaneously mg%	R IV 0.5 g sulfanilamide *per os* mg%	R V 0.5 g sulfanilamide subcutaneously mg%
½	1			2	20
1	1	1	1.5	4	24
2			2.5	5	
3	8	6		5	7
4	8				6
5		6	8	7	6
7		7	10		
24	6			5	6

tions we used 15% sulfanilamide solutions in acetone with addition of glycerine and soap. For the oral and subcutaneous treatment solusceptazine was used. The blood concentration of sulfanilamide was determined at various time intervals following the commencement of treatment.

Results (Table I). The demonstration of sulfanilamide in the blood of animals treated percutaneously proved that the drug penetrates the skin and circulates in the blood. The concentration of sulfanilamide in the blood of animals treated percutaneously (max. 8 mg %) was found to be lower than in those treated subcutaneously (max. 24 mg %), but equal to and possibly higher than in those animals treated orally (max. 7 mg %).

Percutaneous application of sulfanilamide in men: The satisfactory results obtained following the percutaneous application of sulfanilamide in rats and rabbits encouraged us to examine the passage of this drug through the human skin. These experiments were carried out in 14 patients. We employed 15% and 20% solutions of sulfanilamide in acetone with addition of glycerine and soap. The sulfanilamide concentration in the blood was examined 24 hours after inunction and at various other intervals.

Results (Table II). In men the blood concentration of sulfanilamide following percutaneous application is less durable and inferior to that obtained after oral administration of the drug. The blood concentration of sulfanilamide following a single dose of 2 g given orally was less than 1 mg % after ½ hour, reaching a maximum of 1.8 mg % after 4 hours, dropping to 1 mg % after 12 hours and disappearing completely 24 hours after administration. The same dose percutaneously gave a sulfanilamide blood concentration of 1.2 mg % after ½ hour, 1 mg % after 4 hours, not to be found 12 hours

TABLE II.
Blood Concentration of Sulfanilamide after Percutaneous and Peroral Administration in Men.

Sulfanilamide dose in g	Route of administration	½ hr after treatment mg%	2 hr after treatment mg%	4 hr after treatment mg%	12 hr after treatment mg%	24 hr after treatment mg%
2	percutaneously	1.2	1.2	1	0	0
2	oral	traces	1	1.8	1	0
4	percutaneously	1.8	1.4	1.2	1	0
4	oral	traces	1.6	3.2	2	1.2
5	percutaneously	2.0	2.0	1.2	traces	0
10	"	2.7	1.6	1.2	1	0
12	"	3.3	2.0	2.0	1.1	0
16	"	4.0	2.3	2.0	1	0

after administration. On administration of 4 g sulfanilamide *per os*, the concentration of less than 1 mg % after ½ hour rises to 3.2 mg % after 4 hours; the sulfanilamide is still to be detected after 24 hours (1.2 mg %). The same dose percutaneously gives a maximal concentration of 1.8 mg % after ½ hour, 1 mg % after 12 hours, and is not to be detected after 24 hours. Notwithstanding the high single doses employed in the percutaneous application, as high as 16 g, we never encountered a concentration of over 4 mg %. Here, the blood concentration of the drug was highest during the first hour after administration, dropping rapidly during the following 4 hours. No sulfanilamide was found in the blood 24 hours after administration.

Discussion. The penetration of sulfanilamide through the human skin is less perfect than through the rabbit's skin. Thus, the percutaneous route cannot be advised as a general method of administration. This method, however, may frequently render appreciable help as an auxiliary method of treatment in those cases where high doses cannot be administered orally. Patients are frequently unable to keep the sulfanilamide given orally, since it provokes vomiting by local irritation of the stomach or by reaction of the nervous centers. In the former case the subcutaneous or intramuscular route of application is useful, but the low solubility of sulfanilamide does not allow its parenteral administration in high doses. In these cases the percutaneous route provides a way out of the dilemma. For similar reasons, the percutaneous administration of sulfanilamide may be a valuable aid in pediatrics where the injection and ingestion of this drug often meet with difficulties.

In the 14 cases of percutaneous treatment studied, the by-effects encountered were: cyanosis in 4 cases and nausea in only one case. The solvents used (acetone, glycerine, soap) never caused any cutaneous reaction.

Summary and Conclusions. Sulfanilamide if applied percutaneously is resorbed by the skin of rabbits and men. (a) The concen-

tration of sulfanilamide in the blood of rabbits following its percutaneous administration is as high as that obtained after its oral administration, but inferior to the concentration following subcutaneous or intramuscular injections. (b) The blood concentration of sulfanilamide in men following its percutaneous administration is lower than that after oral administration, but vomiting is much more rare and less severe.

The percutaneous use of sulfanilamide may serve as an auxiliary method of chemotherapeutic treatment in certain cases.

13718

Skeletal Abnormalities of Short Spined Turkeys.

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In the course of examining turkey embryos that failed to hatch, mutants with typically shortened necks and bodies (Fig. 1) were found. These embryos varied in that some had an apparently normal head while others had a reduced skull, eyes, and upper beak. None of the affected embryos hatched.

The mutation was found in a closely inbred strain of the Bourbon Red variety of turkeys. Three segregating families gave a ratio of 59 normal to 14 abnormal, which is reasonably close to the numbers expected (54.75 : 18.25) on the basis of a ratio of 3 normal to 1 abnormal. Thirteen short spined and 98 normal embryos were obtained from an untrapnested pen of related birds. Of the 27 short spined embryos the sex was observed for 22, of which 12 were males and 10 were females. The mutation is, therefore, a simple autosomal recessive.

Skeletal Abnormalities. Fully developed embryos were selected for further study from eggs of similar size. The bones were freed from tissue, measured, extracted, dried, and ashed.*

The leg and wing bones of the short-spined embryos are normal in length and thickness (Table I, Fig. 2). The head is likewise normal, whereas the neck and body are much reduced in length.

* I am indebted to Mr. D. C. Stoutamyer and Mr. David Wittenberg, Jr., for preparing the bones and to Dr. H. Goss and the Division of Animal Husbandry for the extractions of the bones.

FIG. 1.
Short spined turkey embryo.

The neck vertebrae are close together but the number of cervical vertebrae, 13 + the atlas, is the same as in normal turkey embryos. The reduction in the length of the body is also due to the crowding together of the vertebrae. There is thus a striking similarity to the short spine mutation of Mohr and Wriedt[1] in cattle.

The leg and wing bones of the short spined embryos had about

TABLE 1.
Measurements on Bones of 29-day-old Turkey Embryos of Approximately Similar Average Weights.

	Avg maximum lengths				Avg widths at middle of bone			
	Normal embryos		Short spined embryos		Normal embryos		Short spined embryos	
Bone or Structure	No.	Length, mm	No.	Length, mm	No.	Width, mm	No.	Width, mm
Femur	12	22.0	4	23.1	12	1.84	4	1.95
Tibia	12	32.4	4	34.8	12	1.94	4	2.00
Tarsometatarsus	12	24.3	4	25.6	12	2.22	4	2.38
Humerus	12	16.6	4	17.9	12	1.63	4	1.38
Ulna	12	15.7	4	15.8	12	1.23	4	1.19
Radius	12	14.6	4	15.3	12	0.73	4	0.74

[1] Mohr, O. L., and Wriedt, C., *J. Genet.*, 1930, **22**, 279.

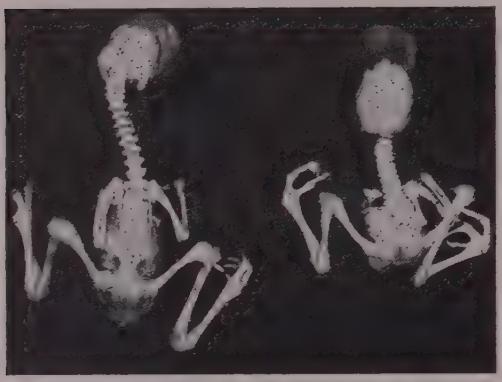

FIG. 2.
Skeletons of 29-day-old turkey embryos (normal—left, short spined—right).

the same dry weight, amount, and percentage of ash as the corresponding bones of normal turkey embryos (Table II). This was also true of the coracoid. The scapula and the ilium had a smaller amount and lower percentage of ash, while the ischium had a lower percentage of ash than the same bones in normal embryos. The pubis of the short spined embryos differed much less from normal than the other pelvic bones.

Discussion. The mutation reported in this paper has an extreme effect on both the gross morphology and composition of a restricted part of the skeleton. It thus differs in its effect on structure from certain mutations such as the creeper which in the homozygote affects all parts of the body (Landauer[2]).

TABLE II.
Average Dry Weight of Bones and Average Ash in Bones of 29-day-old Turkey Embryos.

Bone or Structure	Normal embryos				Short spined embryos			
	No. analyzed	Dry wt in mg	Ash		No. analyzed	Dry wt in mg	Ash	
			Wt in mg	%			Wt in mg	%
Femur	7	40.9	14.2	34.7	4	45.1	15.3	32.8
Tibia	7	69.1	24.5	35.5	4	85.7	27.7	31.9
Tarsometatarsus	7	45.8	15.7	34.3	4	59.9	19.6	33.6
Humerus	7	18.4	5.5	29.9	4	20.3	5.4	26.2
Radius-ulna	7	14.1	4.2	29.8	4	14.1	4.4	31.6
Coracoid	7	8.7	2.4	27.6	4	8.9	2.4	27.6
Scapula	7	6.9	2.7	39.1	4	6.1	1.9	28.3
Ilium	7	19.7	7.9	40.1	4	21.4	6.9	32.2
Ischium	7	7.5	2.6	34.7	4	12.5	2.6	22.5
Pubis	7	2.6	0.8	30.8	4	1.5	0.4	28.6

[2] Landauer, W., *Proc. 7th Internat. Genet. Cong.*

The wing bones of the normal and abnormal 29-day-old turkey embryos had a lower percentage of ash than the leg bones. This agrees with data for embryos of the domestic fowl of comparable age and perhaps indicates that this may be true for embryos of all gallinaceous species and perhaps all Aves. Since Weakley and Dustman[3] found only slight differences between the percentage of ash in the humerus and femur of 5-week-old chicks, the wing bones presumably calcify more rapidly than the leg bones after the birds hatch.

Summary. A simple autosomal recessive lethal mutation that causes crowding together of the vertebrae with consequent shortening of the neck and body is described. The percentage of ash in the wing and leg bones was approximately the same as in the normal embryos, but the percentage of ash was less in the scapula, ilium, and ischium than in the corresponding bones of the normal embryos.

13719

Sex Differences in Pigment Content of Harderian Glands of Mice.

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Recent data[1, 2] have shown that the Harderian glands of female mice of the C_3H strain show two characteristics that differ from those glands found in male mice of the same strain: (1) under normal light the exposed Harderian gland of a female, when stretched upon a microscope slide in a fresh or semidried state, shows a greater degree of "gray" pigmentation, and (2) under ultraviolet light (General Electric BH4) the glands from female mice tend to show a greater intensity of red fluorescence, thus indicating perhaps a higher concentration of porphyrins. (By differential solubilities, HCl numbers and spectroscopic data it has been shown that the porphyrin present in mouse Harderian glands is protoporphyrin.)

[3] Weakley, C. E., Jr., and Dustman, R. B., *J. Agric. Res.*, 1939, **58**, 711; *W. Va. Bull.* No. 294, 40 pages, 1939.

* This experiment has been made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

[1] Figge, F. H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A., *Cancer Research*, 1942, **2**, 335.

[2] Strong, L. C., and Figge, F. H. J., *Science*, 1941, **94**, 331.

In order to verify these observations and to extend the work, the Harderian glands of 468 mice of the C_3H strain have been examined; of these 226 have been from females, 242 from males. These data show that within an inbred strain it appears certain that two variables influence the degree of "gray" pigmentation under normal light. These are (1) sex, and (2) age. When the Harderian glands of litter mates are examined simultaneously it can readily be seen that the females show darker pigmentation than do males. The glands of both sexes appear to diminish in grayness with advancing age. It is also apparent that at a given age the Harderian glands of female mice show more intense red fluorescence than do those from males of the same strain, thus verifying previous observations.

Nine female mice and 9 male mice at 50 days of age were examined simultaneously. The average body weight for the females was 14.66 g; that for the males 17.22 g. The average reading of red fluorescence for the females was 5.0; whereas for the males it was 3.33. Seventeen female mice at 75 days averaged 17.94 g; and 17 males weighed, on the average, 20.23 g. The average reading of fluorescence was: for the females 4.47; for the males 3.11. Twenty-one females at 250 days average 23.2 g; 22 males weighed, on the average, 28.57 g. Average reading of red fluorescene was for the females, 3.14; for the males, 3.08.

At 50 and 75 days (early sexual life) the females showed significantly greater "grayness" than did the males; whereas at 250 days there could be detected no sexual difference.

Glands for the 17 females and 17 males at 75 days of age were extracted separately for porphyrins. The 21 females and 22 males at 250 days were similarly employed. At every step in the following process the fluorescence for the material from the females was more intense than for the males at 75 days of age, but there appeared to be no difference at 250 days.

The glands were ground in sterile sand in a semi-dried condition. The resulting mixture was reddish-tan in color. Glacial acetic acid was added, 1 cc for each mouse (2 Harderian glands). The acid became deeply rose colored—more so for the female glands than for those of the males. Following addition of NaOH until the solution was nearly neutral to Congo red paper, an excess volume of ether was added. The ether was washed 3 times with distilled water, then 1% HCl was added 3 times and finally 10% HCl was added 3 times. All the fluorescent colored material came out from the ether into the 10% HCl solution (following a negative extrac-

tion with 1% HCl solution), thus indicating that the material extracted from the Harderian glands was protoporphyrin. Compared to a standard of coproporphyrin there was approximately 25% more protoporphyrin in the female Harderian gland than in a gland from a male of the same age (75 days) and strain (C_3H).† If a correction for body size be made, this sex difference is even more pronounced.

At 250 days there appeared to be no significant sex difference in the content of protoporphyrins as indicated by the intensity of fluorescence.

Conclusions. 1. The Harderian glands of female mice of C_3H strain at 75 days of life appear to differ in at least two ways from the Harderian glands of male mice of the same age and strain, as follows: (a) They appear to contain under normal light a greater degree of gray pigmentation; (b) they contain approximately 25% more protoporphyrins, in spite of the fact that the glands are smaller and the body weights are also smaller than in males. 2. These differences between sexes are more pronounced at 75 days of age than they are at 250 days.

13720

Experimental Transmission of St. Louis Encephalitis Virus by *Culex pipiens* Linnaeus.*

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Lumsden[1] and Casey and Broun,[2] on the basis of epidemiological investigations, suggested that the virus of St. Louis encephalitis is mosquito-borne. Mitamura and associates[3] in Japan first reported successful transmission of St. Louis virus by a mosquito, *Culex*

† We are deeply indebted to Doctors C. P. Rhoads and K. Dobriner for their kindness in doing this quantitative determination for us.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Lumsden, L. L., unpublished official report, 1933.

2 Casey, A. E., and Broun, G. O., *Science*, 1938, **88**, 450.

3 Mitamura, T., Yamada, S., Hazato, H., Mori, K., Hosoi, T., Kitaoka, M., Watanabe, S., Okubo, K., and Tenjin, S., *Tr. Jap. Path. Soc.*, 1937, **27**, 573.

pipiens var. *pallens* Coq. This has long awaited confirmation and has been regarded with skepticism by many, since various American workers, Leake, Musson and Chope[4] and Fulton, Gruetter, Muether, Hanss and Broun,[5] reported negative results with *Culex pipiens* Linn. Leake and associates[4] and Webster, Clow and Bauer[6] reported negative results with *Anopheles quadrimaculatus* Say; however, the latter found the virus to persist in this mosquito for 42 days, though less than 3 days in *Aedes aegypti* (Linn.). Fulton and associates[5] found it to persist for not more than 10 days in *Culex pipiens*. Hammon, Reeves, Brookman, Izumi and Gjullin[7] reported the isolation of St. Louis virus from naturally infected *Culex tarsalis* Coq. and later confirmed this with 2 other isolations from *C. tarsalis* caught in the same area.[8]

Blattner and Heys[9] have recently transmitted the St. Louis virus under laboratory conditions with *Dermacentor variabilis* (Say), the American dog tick. However, there has been no epidemiological evidence implicating ticks as potential vectors.

Further important evidence supporting transmission by some arthropod is the demonstration of circulating virus in the blood of the monkey (Howitt[10]), the horse (Hammon, Carle and Izumi,[11]), the chicken and the guinea pig (Hammon and Izumi[12]). Thus, virus is available for infection of a blood sucking vector. Serum neutralization tests on animals from the Yakima Valley, Washington (Hammon, Gray, Evans, Izumi and Lundy[13]) showed that a number of domestic and wild birds and mammals had high antibody titers to this virus. It appears probable, therefore, that the potential reservoir for infection of an arthropod vector is large.

A serious difficulty is encountered in any attempt to transmit this

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4 Leake, J. P., Musson, E. K., and Chope, H. D., *J. A. M. A.*, 1934, **103**, 728.

5 Fulton, J. D., Gruetter, J. E., Muether, R. O., Hanss, E. B., and Broun, G. O., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 255.

6 Webster, L. T., Clow, A. D., and Bauer, J. H., *J. Exp. Med.*, 1935, **61**, 479.

7 Hammon, W. McD., Reeves, W. C., Brookman, B., Izumi, E. M., and Gjullin, C. M., *Science*, 1941, **94**, 328.

8 Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., *J. Infect. Dis.*, in press.

9 Blattner, R. J., and Heys, F. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **48**, 707.

10 Howitt, B. F., *J. Immunol.*, 1941, **42**, 117.

11 Hammon, W. McD., Carle, B. N., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 335.

12 Hammon, W. McD., and Izumi, E. M., unpublished data.

13 Hammon, W. McD., Gray, J. A., Evans, F. C., Izumi, E. M., and Lundy, H. W., *Science*, 1941, **94**, 305.

disease experimentally since the usual laboratory animals fail to develop a fatal encephalitis following subcutaneous inoculation of such a dose of virus as might be expected from a mosquito bite. This probably explains some of the negative results obtained by others. To circumvent this difficulty we have used special methods including isolation of the virus from the brain or blood of the vertebrate host during the course of the inapparent infection, and virus neutralization tests on a preliminary and post-experimental serum specimen.

Culex pipiens, collected as adults, were infected by feeding on a 10% brain suspension of St. Louis virus in 5% sheep serum infusion broth mixed with equal parts of whole defibrinated rabbit blood. A cotton pad was soaked with this suspension, sprinkled with sugar, and the mosquitoes were allowed to engorge upon it. The virus was a strain which had been through relatively few mouse brain passages since isolation from naturally infected mosquitoes.[7] All mosquitoes which had engorged on the infective suspension were held until used at 23° to 27°C.

Four experiments were performed, 3 of which yielded positive results. In each of the first 2, infected mosquitoes were allowed to feed repeatedly on a pigeon whose blood had been demonstrated to be free from antibody. Feedings were begun 5 days after the infective meal. In one experiment there were 111 feedings and in the other, 30. Fifteen days after the last mosquito fed each pigeon was found to have developed a high titer of serum neutralizing antibody. All mice used in the neutralization test survived both dilutions of virus used.[14] A normal pigeon kept in the same cage as the 2 experimental birds, but not subjected to mosquito feeding, did not develop protective antibodies during the experimental period. Neither of the pigeons showed visible clinical signs as a result of infection.

In the next experiment, the results of which are presented in Table I, 100 blood-virus-engorged mosquitoes were employed. They were permitted after various intervals to feed on doves and on baby mice of less than 8 days of age. Virus was isolated from the blood of one dove 24 hours after feeding and from the brain of one mouse. Antibodies were demonstrated to have developed in the serum of 3 doves and not in the control. Several of the doves appeared to be ill.

In a fourth experiment only 30 mosquitoes engorged. Baby mice were used, but a total of only 25 mosquito feedings occurred. No virus was demonstrated in the brains of these mice, or from the mosquitoes tested.

In all experiments samples of mosquitoes were removed at varying

[14] Hammon, W. McD., and Izumi, E. M., *J. Immunol.*, 1942, **43**, 149.

TABLE I.
Results of Third Transmission Experiment with *Culex pipiens*.

Days after infective meal when fed on vertebrate	Virus test on dove's blood*	Virus test on baby mouse's brain†	Neutralization test on dove's blood	Virus test on mosquito suspension
Control dove	—	—	0	—
4	0 (20)‡	+ (10)	++	—
6	0 (21)	0 (15)	0	+ [30]§
8	0 (15)	0 (6)	+	—
10	—	0 (10)	—	—
11	+ (15)	0 (5)	+	—
13	—	0 (5)	+	+ [20]

*Blood tested 24 and 48 hours after mosquitoes fed.
†Baby mice were exhausted by mother after mosquito feeding, then sacrificed 5 days later and brain tested for virus.
‡( ) Number of mosquitoes which fed.
§[ ] Number of mosquitoes ground up in suspension.

intervals of from 3 to 18 days after the infective meal, and virus was isolated at 3, 6, 7, 9, 12, 13, and 15 days. Only one sample was tested after a longer period of incubation (14 mosquitoes at 18 days) and this was negative. A few samples taken earlier were also negative.

Summary and Conclusions. It appears that certain species of *Culex* mosquitoes may act as vectors of the virus of St. Louis encephalitis. This is supported by the following evidence: 1. In certain areas *Culex* mosquitoes have fitted into the epidemiological picture to the extent that several workers have stressed their probable importance as vectors. 2. Virus is available to mosquitoes in the blood of doves and a number of other species of experimentally infected vertebrates. Evidence of naturally acquired infection has been demonstrated in the serum of many vertebrate hosts. 3. Infected *Culex tarsalis* have been collected in nature. 4. *Culex pipiens* has been shown to act as an experimental vector, transmission having been demonstrated to occur between the 4th and the 11th day after an infective meal. Virus persists in the mosquito for at least 13 days.

13721

A New Method for Concentrating Influenza Virus from Allantoic Fluid.

G. K. Hirst, E. R. Rickard and L. Whitman. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division of The Rockefeller Foundation, New York.

In a recent communication from this laboratory[1] a number of different influenza vaccines were described, and their relative antigenicity in man was compared. The highest mean post-vaccination antibody levels were obtained with those preparations which because of concentration contained the greatest amounts of virus. The concentrated virus was obtained from allantoic fluid by centrifugation at 11,000 rpm for 2 hours. In the present paper another method of virus concentration is described, together with the data showing the antigenic potency of such concentrates when given to man.

Preparation of Virus Suspensions. All of the virus suspensions used, both for concentration experiments and for testing of sera, were prepared from the allantoic fluid of eggs infected with either the PR8 strain of influenza A virus[2] or the Lee strain of influenza B virus.[3] The method of preparation used was described in the previous report on the series of vaccinations.[1]

Virus titrations in mice, measurements of red cell agglutination titer, and agglutination inhibition antibody titrations in human sera were all done as described in the previous report.[1] The only change in the method was that the degree of agglutination in the red cell tests was determined by means of a photoelectric cell rather than by comparison with visual standards. In order that the antibody levels of the human sera in this series might be compared directly with those previously reported, the same standard ferret immune sera for each virus (PR8 and Lee) which had been used as controls in the former tests were again titrated. Seven duplicate titrations of each standard serum were distributed throughout the tests as a check on the uniformity of the technic.

We repeatedly observed that when allantoic fluid from 13-day-old chick embryos, both normal and infected, was frozen and thawed,

1 Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1942, **75**, 495.

2 Francis, T., Jr., *Science*, 1934, **80**, 457.

3 Francis, T., Jr., *Science*, 1940, **92**, 405.

a fluffy white precipitate appeared in the suspension. This precipitate usually went back into solution when the fluid was warmed to 37°C. The amount of precipitate varied but was of the order of 0.2 to 0.5 mg per cc of fluid and consisted of both protein material and amorphous urates.

In addition, another type of precipitate occurred in allantoic fluid after prolonged storage at −10° or at 4°C. This precipitate was insoluble even when warmed above 37°C; it consisted mainly of urates. This insoluble precipitate did not possess the properties which will be described for the soluble sediment.

A suspension of PR8 virus in allantoic fluid, which had been stored for several weeks at −72°C, was thawed, and the precipitate which formed was removed by centrifugation at low speed (1000 rpm) before the temperature of the fluid had risen appreciably. The supernatant fluid was removed and the sediment resuspended in one-tenth the original volume, the supernatant fluid being used as diluent. Samples of this suspension were taken immediately after thawing. Together with the supernatant fluid and the resuspended sediment they were tested in mice for lethal virus titer and *in vitro* for agglutination titer. The results recorded in the first part of Table I show that approximately 90% of the virus originally present in the suspension was removed with the precipitate and that a large part of the removed virus could be accounted for by the titer obtained with the concentrated sediment. The *in vitro* and *in vivo* tests gave corresponding results. This experiment has been repeated a number of times, and 90 to 95% of the virus was usually carried down with the sediment, as judged from *in vitro* titrations.

Similar attempts to concentrate the virus with suspensions of the insoluble type of precipitate failed, and amorphous urates did not show the phenomenon. The factor responsible for bringing down the virus was soluble at 37°C.

When the same technic was applied to the concentration of the Lee strain, the results varied and usually only about one-half of the total virus was removed with the precipitate. It was found, however, that when a recently thawed suspension was changed from the usual pH of 8.0 to 6.6 or 6.7, a higher proportion of the virus was adsorbed on the precipitate. Separation of the sediment by low speed centrifugation at a pH of 6.7 usually removed 75% of the virus originally present. Reducing the pH to this level did not detectably destroy any of the virus activity. The results of concentrating a Lee virus suspension at pH 6.7 are given in Table I.

In order to test the antigenic potency of influenza virus concen-

TABLE I.
Concentration of Influenza Virus on the Precipitate of Freshly Thawed Allantoic Fluid.

Strain		Fluid before concentration	Suspension of precipitate concentrated 10 ×	Supernatant after removal of precipitate
PR8	50% mouse mortality titer	$10^{-4.1}$	$10^{-4.8}$	$10^{-3.2}$
	Agglutination titer	275	2900	26
Lee	50% mouse mortality titer	$10^{-3.6}$	$10^{-4.6}$	$10^{-2.7}$
	Agglutination titer	64	1024	14

trated in this way, the following vaccine was prepared and given to human beings.

Preparation of Vaccine 66. One liter of fluid containing PR8 virus was carefully thawed, and the precipitate which formed was removed by centrifugation at 1000 rpm for 10 minutes. The temperature of the fluid did not rise appreciably above 0° during this process. The sediment was resuspended in 100 cc of the supernatant fluid and formalin added to a final concentration of 0.4% formaldehyde. The suspension stood overnight at 4°C. One liter of fluid containing Lee virus was similarly treated, except that the pH was adjusted to 6.7 by the addition of acetic acid, before the precipitate was removed. Care was taken to keep the temperature of the suspension close to 0°C until the sediment and supernatant were separated. The precipitate was resuspended in 100 cc of supernatant and formaldehyde added to a final concentration of 0.4%. After 18 hours the two concentrates were combined and homogenized in a Waring mixer for 3 minutes. The mixed suspension was distributed in ampoules in 25 cc amounts, frozen, and dried.[4] Each ampoule was rehydrated with 50 cc of saline just before use. Each person received a subcutaneous injection of 2 cc, which contained 20 mg of dried material. On rehydration much of the dried material proved to be insoluble but formed a fine suspension which was administered without difficulty.

Virus titrations in mice on the tenfold concentrated suspensions before formalinization gave a 50% mortality titer of 10^{-6} for the PR8 suspension and $10^{-4.7}$ for the Lee suspension. The corresponding agglutination titers were 4000 and 2000, respectively. There was no detectable active virus in the finished vaccine.

This preparation was given to 100 individuals. Each person received the concentrate from 5 cc of PR8 and 5 cc of Lee allantoic fluid, and the number of 50% mouse mortality doses injected is

[4] Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1940, **71**, 83.

TABLE II.
Antibody Response of Human Beings to Injections of Influenza Viruses Prepared from Allantoic Fluid.

Strain	Vaccine No.	No. of 50% mouse mortality doses per person	Mean pre-vaccination antibody titer	Mean 2-wk post-vaccination antibody titer	No. individuals tested
PR8	55	362,000	168	461	140
	57	3,620,000	163	935	120
	66	10,000,000	114	1300	100
Lee	55	10,000	87	276	140
	57	40,000	84	443	120
	66	500,000	81	680	100

Vaccine 55, viruses unconcentrated and active.
Vaccine 57, PR8 virus concentrated tenfold and Lee virus concentrated fourfold by centrifugation. Viruses active.
Vaccine 66, viruses concentrated tenfold on precipitate; 0.4% formaldehyde added.

shown in Table II. The geometric mean of the pre- and postvaccination antibody levels is also given in this table. For purposes of direct comparison, similar data are given on Vaccines 55 and 57, which were described in the previous report.[1] Vaccine 55 contained unconcentrated active virus in allantoic fluid, while Vaccine 57 was made by concentrating the virus from infected fluid by high speed centrifugation (11,000 rpm for 2 hours). The virus in Vaccine 57 was also active.

From Table II it may be seen that the antibody levels following the injection of Vaccine 66 were higher for both the PR8 and the Lee strain than the corresponding mean values obtained with Vaccine 57. These higher levels were doubtless due in part to the larger amount of virus in Vaccine 66. One may safely conclude, however, that the two preparations elicited antigenic responses which were at least of the same order of magnitude, in spite of different methods of virus concentration and the fact that Vaccine 57 contained active virus while Vaccine 66 was formalinized. Vaccine 55 was included to emphasize again the wide disparity in the results obtained with concentrated and unconcentrated preparations. These results fortified our earlier conclusions[1] that the amount of virus injected is the most important single factor in determining the antibody level obtained and that inactivation by formaldehyde does not have a significantly adverse effect on the antigenicity of influenza virus for man.

The formation of a precipitate in freshly thawed allantoic fluid has been described, and it has been shown that under certain conditions a considerable amount of influenza virus may be adsorbed on this precipitate. This phenomenon provides a method for concentrating the virus from allantoic fluid on a large scale which is not

technically difficult and can be done with ordinary laboratory equipment. PR8 and Lee viruses were concentrated from allantoic fluid by this method, and human beings were inoculated with a formalinized concentrate. The mean antibody level of the subjects 2 weeks after vaccination was at least as high as that which followed the injection of similar amounts of active virus concentrated by centrifugation.

13722

Use of Biotin for Stimulating Growth of Nerve Tissue and Other Cells *in vitro*.

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A rapidly increasing number of data show that biotin is of widespread metabolic significance for a wide range of living things, and the fact that this vitamin markedly increases the growth rate of yeast cells and bacteria indicates that it might have similar effects on other plant or animal cells. Our present limited knowledge about many of the viruses, particularly those of the neurotropic group, is due in large part to the difficulties encountered in trying to grow them *in vitro*. In view of the important relationship between the metabolic state of cells and the ability of viruses to infect them,[1] experiments were devised to test the effects of biotin on various cell types, especially nerve tissue, in an effort to apply the data thus obtained for initiating or improving the growth of viruses and rickettsiae *in vitro*.

The method used for studying the effects of biotin on living cells was a slight modification of a technic previously described, using hanging drop cultures.[2] A piece of tissue was removed, preferably from the midline of an embryo, and divided into two equally-sized (and usually, bilateral) halves. One piece was explanted into a clot made up of plasma, embryonic extract, and biotin in Tyrode solution. A control culture was made of the other half in a clot consisting of the same proportions of plasma, extract, and plain Tyrode solution. Since the biotin (obtained from the S. M. A. Corporation) was furnished in alcoholic solution, it was necessary to add an

1 Plotz, H., *C. R. Soc. Biol.*, 1937, **125**, 603, 719.

2 Hamilton, H. L., *J. Exp. Zool.*, 1941, **88**, 275.

equivalent amount of alcohol to the Tyrode solution used in control cultures. In most of the concentrations (1/6 γ to 5/6 γ of biotin per cc), the amount of alcohol was too small to affect the results appreciably. Explants consisted of brain, spinal ganglia, skin, mesenchyme, and somite material from 4- to 7-day chick embryos and from mouse embryos of various ages up to parturition. Chicken plasma and chick embryonic extract were used in all cultures. The pairs of treated and control cultures were incubated at 37.5°C for 4 to 6 days, and the comparative amounts of growth were measured.

In a total of 106 pairs of explants (both chick and mouse) 65% showed a stimulation of growth of cells in the presence of biotin. Within certain limits, the amount of stimulation was in proportion to the concentration used. For example, in one series of explants, using chick tissue, the average increase in outgrowth produced by biotin cultures over controls during a 4-day period was 358 μ for a concentration of 1/6 γ per cc and 453 μ for 1/3 γ per cc. The increase (442 μ) obtained with 5/6 γ per cc was not in proportion to the concentration, possibly because of inhibition by the alcohol in the biotin solution, since the control cultures, containing the same amount of alcohol, did not show as good growth as ordinary control cultures. Among the concentrations used, the optimum amount of growth occurred with 1/3 γ of biotin per cc (final concentration in the clot). Increased outgrowth was observed in epithelial cells, fibroblasts, muscle cells, and nerves.

A detailed study was made of the effects of biotin on nerve tissue, since the amount of growth obtained heretofore has been of limited extent and duration (See Levi for a résumé of this problem).[3] In addition to an increase in axon or fiber regeneration, which was often 3 or 4 times that obtained in control cultures at a concentration of 1/3 γ per cc, active proliferation of nerve cell bodies occurred in some cases, apparently depending on the region of the brain which was sampled. True mitotic division, with the formation of spindle and chromosomes, was not observed, but there was much amitotic activity, resulting in the formation of a narrow zone of outgrowth.

These effects of biotin are apparent only on embryonic nerve tissue, before the primordia are completely differentiated. For example, fiber growth was obtained from all parts of brains of mouse embryos, but only from the cerebral hemispheres of a newborn mouse, and none at all from any part of a juvenile mouse brain. A similar progressive fixation was observed when attempts were made to transfer and subculture nerve cells. Active proliferation of

[3] Levi, G., *Zeit. f. d. Gesamte Anat.* (Erg. Anat.), 1934, **31**, 125.

cell bodies was observed only in the original cultures. Fiber regeneration occurred in four successive subcultures, made over a period of 25 days, but the extent of the outgrowth diminished with each passage. The decreased size of the explant with each transfer may be a partial cause for this latter effect, since a portion of the tissue is invariably lost at each transfer.

It may appear somewhat surprising that biotin in such dilute concentrations should have a marked effect on the proliferation of embryonic cells, in view of the large amounts of biotin known to be present in eggs.[4] Presumably, the chick embryos, from which the embryonic extract was prepared, should contain quantities of biotin, unless the vitamin is used up by the cells as fast as it is absorbed from the yolk. However, it is not unprecedented to find that very minute amounts of biotin can produce a considerable effect. For example, Du Vigneaud, *et al.*,[5] find that, in rats, the addition of 2-4 γ per day of biotin to an ordinarily protective diet was sufficient to increase greatly the incidence of tumors produced by the carcinogenic compound, "butter yellow."

In view of the fact that biotin is actively synthesized by many species of bacteria and molds and is apparently important in their nutrition,[6] it is hoped that the vitamin may prove valuable in producing higher titers of virus or larger yields of rickettsiae in culture. Experiments are in progress to test these possibilities.

13723

Protective Action of Desoxycorticosterone Acetate and Progesterone in Adrenalectomized Mice Exposed to Low Temperatures.

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The early work of Wyman and Tum Suden[1] with autoplastic trans-

4 Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

5 DuVigneaud, V., Spangler, J. M., Burk, D., Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, 1942, **95**, 174.

6 Landy, M., and Dicken, D. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 449.

* This work was started at the Roche-Organon Laboratories, Nutley, New Jersey.

1 Wyman, L. C., and Tum Suden, C., *Am. J. Physiol.*, 1929, **84**, 362.

plants of cortical tissue in adrenalectomized rats indicated a rôle of the adrenal cortex in the maintenance of body temperature. This work was followed by the use of cortin in preventing a drop in colonic temperature of adrenalectomized rats (Hartman, *et al.*[2]). Recently it was shown that corticotropin could protect hypophysectomized but not adrenalectomized rats against cold.[3]

Widström,[4] and Selye and Schenker[5] suggested the use of the cold exposure test as a quick and accurate method for the bioassay of cortin. The latter used adrenalectomized rats (35-50 g) and 12 to 24 hours after operation exposed the animals to a low temperature. Treatment was started at the time of exposure and was spaced at 2-hour intervals. By this technic they were able to detect amounts as small as 0.03 cc of cortin (Wilson preparation). In view of this action of cortin and the sensitivity of the test, it seemed warranted to study the influence of desoxycorticosterone acetate (D C A).† A modification in technic was employed. This modification consisted in treating the adrenalectomized mice previous to their exposure to cold. The D C A was dissolved in an oil vehicle instead of water.

Experimental. Young adult female mice were adrenalectomized and on the same day received the first of 4 injections of D C A or control substance. The following day they received the next 2 injections, and on the third day, the fourth injection was given. Three groups of mice received D C A in doses of 100 γ, 250 γ and 500 γ, each dose being dissolved in 0.1 cc of peanut oil. The total amount of hormone administered was 0.4, 1.0, and 2.0 mg respectively, per mouse. Of 4 other groups of adrenalectomized mice, one received no treatment, one received 500 γ of progesterone, and a third and fourth were given 0.1 and 0.2 cc of peanut oil per injection.

One hour after the last injection, the mice were put into small wire mesh cages and placed in either a refrigerator or cold room at $6 \pm 2°$C. The experiment was further controlled by subjecting both normal, untreated adrenalectomized, and sham operated mice to cold. The animals were observed hourly and the number and percentage of survivors noted.

Discussion. Preliminary experiments, using the Selye-Schenker

[2] Hartman, A. A., Brownell, K. A., and Crosby, A. A., *Am. J. Physiol.*, 1931, **98**, 674.

[3] Tyslowitz, R., and Astwood, E. B., *Am. Physiol. Proc.*, 1941, **133**, 427.

[4] Widström, G., *Acta Med. Scandinav.*, 1935, **87**, 1.

[5] Selye, H., and Schenker, V., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 518.

† The hormones used were prepared by Roche-Organon.

procedure which involves treatment during exposure to cold, gave negative results for desoxycorticosterone acetate dissolved in oil. For this reason the precold treatment schedule was used and its success can probably be explained on the basis of slower absorption of D C A from oil than cortin from a water solution. Furthermore, the new procedure was designed to give the mice time to recover from the shock of the operation.

It was observed that in the cold-exposure test adult animals could be used as readily as immature ones. Apparently removal of a major portion of the adrenal tissue is sufficient to insure death in 100% of the mice within 6 hours after exposure to low temperature; whereas, at room temperature a number were found to survive indefinitely. These showed adrenal rests at autopsy. The average life span of successfully adrenalectomized mice in the strain used in the present report is about 5 days, which agrees with the figure of 3 to 7 days given by Pfeiffer and Hooker.[6]

The mortality curves (Fig. 1) show little protection by D C A at the dose level of 100 γ per mouse, but at 250 γ and 500 γ excellent protection was observed. It is probable that a dose smaller than 250 γ but greater than 100 γ would also give significant protection. Pfeiffer and Hooker[6] showed that 250 γ of D C A could maintain life in adrenalectomized mice at room temperature. Since a daily dose of

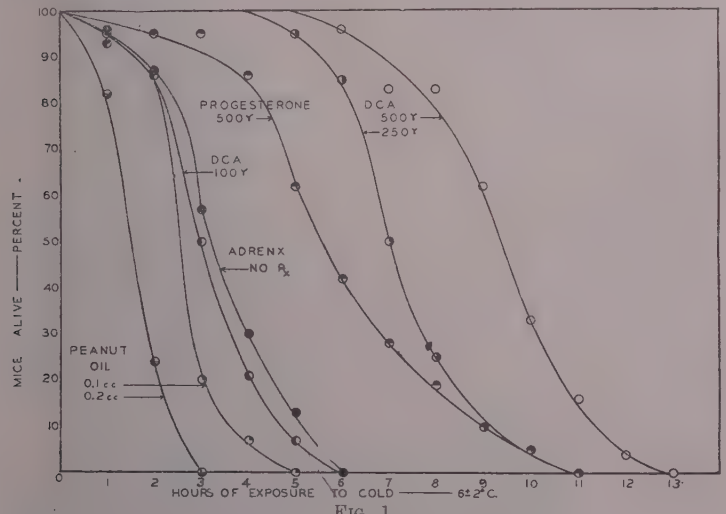

FIG. 1.

Mortality curves of adrenalectomized mice exposed to cold (6 ± 2°C). A total of 179 mice were used and each curve is based on 17 to 24 mice.

[6] Pfeiffer, C. A., and Hooker, C. W., *Am. J. Physiol.*, 1940, **131**, 441.

1 mg of D C A is required to maintain the adrenalectomized rat[7] and 0.5-1.0 mg to maintain the adrenalectomized guinea pig[8] at room temperature, and since the entire cold exposure test requires 3 days, this procedure offers certain advantages with respect to sensitivity and time.

It is a known fact that progesterone can prolong the life of adrenalectomized animals. In the present experiment, progesterone was tested at the dose level of 500 γ (Fig. 1) and a mortality curve obtained somewhat similar to the D C A curves. Comparison of the protective action of progesterone and D C A at the 50% survival level indicates a ratio of 2.6 to 1.

Kendall[9] stated that compounds with an oxygen atom on C_{11} and those found in his amorphous fraction can maintain life in adrenalectomized rats subjected to low temperatures. He found that corticosterone and compound E were about equally active, namely, at dose levels of 16 γ and 20 γ respectively. The present data indicate that D C A also has a protective action, but at a dose level of 250 γ. This is a much greater amount than that of the former compounds and while it may be explained on the basis of a difference in action, *i. e.*, corticosterone is more effective in glycogen metabolism and D C A, in electrolyte metabolism, in this case it would appear that the difference is quantitative and not qualitative.

Control experiments indicate that peanut oil has a toxic effect. Mice receiving 0.2 cc of peanut oil showed a significant decrease in resistance to cold as compared with the untreated adrenalectomized mice. Beznak and Korenyi[10] reported that arachis oil increased the mortality of a "working rat population," and Bruce and Tobin[11] demonstrated the presence of a fraction in sesame oil that was toxic to adrenalectomized rats.

Summary. By the use of a precold treatment injection schedule it has been demonstrated that desoxycorticosterone acetate at dose levels of 250 γ and 500 γ and progesterone at 500 γ prolongs the life of adrenalectomized mice subjected to low temperatures. Peanut oil at the dose level of 0.2 cc has been shown to possess a significant toxic action.

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7 Grollman, A., *J. Pharm. and Exp. Therap.*, 1939, **67**, 257.

8 Clark, W. G., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 253.

9 Kendall, E. E., *Proc. Staff Meet. Mayo Clin.*, 1940, **15**, 297; *J. A. M. A.*, 1941, **116**, 2394.

10 Beznak, M., and Korenyi, A., *Arch. int. Pharmacodyn.*, 1941, **45**, 321.

11 Bruce, R. A., and Tobin, C. E., *Endocrinol.*, 1940, **27**, 956.

13724 P

Influence of Hypophysectomy on Epithelization of Wounds and on Fibroplasia.

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In the present studies, attempts are made to determine what effects the removal of the hormones of the pituitary and of those glands under its control has upon the ability of an animal to exert reparative epithelial growth or reparative fibroplasia.

Rats of the Wisconsin strain, obtained from the local Anheuser-Busch laboratories were used. At the age of approximately 14 weeks they were hypophysectomized, and then used 7-10 weeks later, at which time they showed a 10-20% loss of weight, loose thin skin, emaciation, appearance of fine silky hair, atrophy of testes, and atrophy of the adrenals, gonads and thyroid at postmortem.

In the first part of the experiment an area of 2 sq cm was resected from the right flank of 19 normal and 18 hypophysectomized animals under nembutal-ether anesthesia. This area was measured on the first and every other subsequent day by tracing on cellophane and measuring with a planimeter. The reduction in wound areas for the hypophysectomized animals is identical with that of the controls.

The wounds in the normals were slightly larger than those in the experimentals for the first 4 days. This is probably due to a greater amount of elastic tissue and less stretching of the normal skin before the area was demarcated, for in the normals the skin is more tightly bound down by the subcutaneous tissue that is strikingly lacking in the hypophysectomized animals.

In the second part, we attempted to test the rate of fibroplasia. Harvey had shown that fibroplasia is proportional to the return of strength in wounds of the stomach. Using his technic, incisions 1 cm long were made in the stomachs of 29 normals and 18 hypophysectomized rats. These were sutured with a running mattress suture of 000 catgut. At varying intervals postoperatively, the animals were sacrificed, a cannula inserted through the pylorus, the esophagus and duodenum tied off, and air introduced into the stomach. The system was connected to a mercury manometer and a pressure rise of 100 mm mercury per minute was maintained. The mercury level reached when the wound broke was regarded as indicating the relative amount of fibroplasia in the wound.

The return of strength occurred from the 4th to the 8th day. In this part of the curves the rise is most rapid, and the two curves are similar. On the 10th day, in many instances, the stomach broke before the wound did—a phenomenon observed by Harvey on the 10th and 12th days. In the initial 2 days, the wounds of the experimental animals were stronger than the normals. We are unable to explain this.

Hypophysectomy resulted in no change in the rate of epithelization of skin wounds or fibroplastic repair of stomach wounds in the rats.

13725

Effect of Saline Infusions Upon the Blood Volume and Serum Proteins of Hypoproteinemic Dogs

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Calvin[1] reported that the total circulating proteins of normal dogs could be increased by relatively large infusions of normal saline. During the course of other experimental work[2] it seemed pertinent to study the effect of massive infusions of saline upon the total amount of circulating protein in both normal and hypoproteinemic dogs.

Three dogs in varying states of nutritional hypoproteinemia and one normal dog were used in this work. After a preliminary determination of blood volume and serum proteins had been made, the experimental animals were given relatively large intravenous infusions of normal saline solution during short periods of 6 to 9 minutes. The blood volumes and proteins were studied in these animals at varying intervals. The methods used for determining blood volume, serum proteins and non-protein nitrogen have been described.[2]

The data for the plasma volumes and serum proteins are presented in Table I. The total circulating protein is calculated from plasma volume and serum protein concentration. After relatively large saline infusions the absolute amount of circulating protein in the hypoproteinemic animals shows practically no change during the

1 Calvin, D. B., Proc. Am. Physiol. Soc., *Am. J. Physiol.*, 1939, **126**, 454.

2 Shearburn, E. W., *Surg., Gynec., and Obst.*, 1942, **74**, 343.

TABLE I.

Time after infusion, min	Plasma volume, cc	Total serum protein, g%	Total circulating protein, g	Time after infusion, min	Plasma volume, cc	Total serum protein, g%	Total circulating protein, g
Dog XI—Normal				Dog I—Hypoproteinemic			
Control	746	5.70	42.5	Control	445	4.25	18.9
350 cc Saline Injected				250 cc Saline Injected			
30″	963	4.80	46.2	30″	511	3.76	19.2
360″	785	5.68	44.6	360″	522	3.90	20.4
Dog XI—Normal				Dog V—Hypoproteinemic			
Control	700	5.62	39.3	Control	533	3.70	19.7
250 cc Saline Injected				200 cc Saline Injected			
30″	902	5.01	45.1	30″	626	3.20	20.0
360″	723	5.54	40.0	360″	580	3.52	20.4
Dog XI—Normal				Dog VIII—Hypoproteinemic			
Control	764	6.02	46.0	Control	429	5.38	23.2
350 cc Saline Injected				250 cc Saline Injected			
15″	1160	4.60	53.4	15″	579	3.79	22.0
360″	822	6.03	49.6	360″	552	4.48	24.7

first thirty minutes and at the end of 6 hours there is a slight but definite increase. On the other hand, infusions in normal animals are followed immediately by a relatively large increase in the circulating protein. At the end of 6 hours, total circulating protein decreases but does not return to the preinfusion level.

In 3 different experiments the maximum increases in total circulating protein in a normal dog were 8.7, 15, and 16% respectively at 30 minutes. The maximum increases in total circulating protein in hypoproteinemic dogs were 3.5, 6.5, and 8.0% respectively, at the end of 6 hours.

To summarize, large injections of saline in the normal dog result in an immediate increase in the total circulating protein. Apparently the labile protein stores of the normal dog enter the blood stream quite rapidly in an attempt to maintain an osmotic equilibrium. When large saline infusions are given to hypoproteinemic dogs, there is a smaller increase in the total circulating protein. Since the protein reserves are depleted, the protein influx into the peripheral circulation is considerably less and occurs more slowly.

13726 P

Ionization of Sulfonamides.

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A recent quantitative analysis of bacteriostasis by sulfanilamide, sulfapyridine, sulfadiazine and sulfathiazole showed that for inhibition of *Es. coli* decreasing amounts of each drug in the order named were required.[1] Actually over six hundred times more sulfanilamide is required than sulfathiazole or sulfadiazine, and similar results have also been obtained with a variety of other microörganisms,[2] indicating that this regular increasing order of bacteriostatic potency is characteristic of these N_1 sulfonamides (Col. A, Table I) and not attributable to the selective action of one drug against a certain organism. Furthermore, the quantitative relationships between these sulfonamides and para aminobenzoic acid (PAB) differ just as widely; *e. g.*, over 600 times more PAB is needed to block sulfathiazole and sulfadiazine than to block sulfanilamide. (Col. F.) When the minimum effective concentration of each drug was tested, the same amount of PAB was required to block each drug. (Col. E.) In other words, going from sulfanilamide to sulfadiazine a progressively larger proportion of each drug actually present in the cultures appears to possess activity against either bacteria or PAB; the active moiety presumably being similar for each drug.

It seems possible that such a partition into active and inactive portions might be controlled by the extent to which the drugs are ionized in the cultures. Accordingly the dissociation constants were measured* (Col. B) and the percent of each drug ionized at pH 7.0 (Col. C) was computed. (Some shift in the apparent pK may be expected to occur in complex biological media.) Based on these values and using the minimum effective amounts of drug added to the culture (Col. A), the concentration of ionized drug was estimated

1 Rose, H. M., and Fox, C. L., Jr., *Science*, 1942, **95**, 412.

2 Fox, C. L., Jr., and Rose, H. M., unpublished data.

* The drugs were dissolved in CO_2 free water and titrated with 0.01 N NaOH using the Beckman glass electrode and potentiometer. The pK_A values were also obtained by measuring the pH of equimolar mixtures of the drugs and their sodium salts in CO_2 free water. The drug concentrations used were about 0.001 M.

TABLE I.
Ionization of Sulfonamides and Effective Concentrations of the Drugs.

	(A) Min. effective drug conc., M × 10⁻⁶	(B) Acid dissociation constant, pK_A	(C) % ionized at pH 7.0	(D) Conc. of ionized drug pH 7.0 M × 10⁻⁶	(E) Min. amt PAB required to prevent bacteriostatis with drugs, Col. A, M × 10⁻⁶	(F) PAB / Drug Ratio	(G) PAB / Ionized drug (Col. D)
Sulfanilamide	2500	10.5	0.03	0.71	0.5	1-5000	1/1.4
Sulfapyridine	20	8.5	3.4	0.68	0.5	1-40	1/1.4
Sulfathiazole	4	6.8	61.6	2.46	0.5	1-8	1/4.9
Sulfadiazine	4	6.4	80.0	3.2	0.5	1-8	1/6.4

The pK_A of para aminobenzoic acid is 4.9.[8,9] Its influence on the ionization of these drugs is under investigation.

(Col. D). From these values, the ratios PAB/Ionized Drug were obtained (Col. G).

A comparison of Cols. B and A shows that the variations in the dissociation constants and in the minimum effective concentrations are in the same order. More significant, however, is the fact that the concentration of ionized drug is approximately the same in cultures containing very different amounts of these drugs. Comparison of Cols. F and G shows that the wide differences in the PAB/Drug ratios are sharply narrowed and the ratios PAB/Ionized Drug are of the same order of magnitude.

From these data certain important inferences may be drawn. It appears that only the ionized portions of the sulfonamide acids exert antibacterial action. Furthermore, the minimal amount of each drug (ionized portion) needed to obtain bacteriostasis is approximately the same. In other words it is possible that the relative bacteriostatic potency of these drugs is only a measure of their ionization at biological hydrogen ion concentrations. To test this inference the minimal bacteriostatic concentration of sulfanilamide was measured at pH 6.8 and at pH 7.8. Although growth in the controls was the same at both pH levels, 20,000 μM per L of drug were required for bacteriostasis at pH 6.8, whereas only 2,500 μM per L were needed at pH 7.8. Since the ionization of sulfanilamide at pH 7.8 is increased ten-fold over that at pH 6.8, the amounts of ionized drug in the cultures are actually similar, despite the widely differing total amounts of drug employed. The significance of these observations in terms of a mechanism of sulfonamide action needs further study. Woods postulated competition for a bacterial

[8] Holmberg, *Z. f. Phys. Chem.*, 1908, **62**, 728, cited in Beilstein, 1931, **14**, 419.
[9] Albert, A., and Goldacre, R., *Nature*, 1942, **149**, 245.

enzyme between one mol of an essential metabolite, PAB, and 5-25,000 mols of sulfanilamide.[3] It is apparent from the previous experiments[3] that the quantity of enzyme (number of bacterial cells) is irrelevant. The present data suggest that all the drugs react in roughly similar amounts with approximately equivalent quantities of PAB.

There are, in addition, important physiological implications. Since at the pH of body fluids sulfanilamide on the one hand is almost completely unionized and sulfadiazine is almost completely ionized, the question might well be raised as to the relative effectiveness of similar blood levels of these drugs. There is a possibility that in therapy lower blood levels of sulfathiazole and sulfadiazine might be just as effective since concentrations in excess of the minimum effective level do not further retard bacterial growth.

This difference in degree of ionization also exercises considerable control over the solubility of these drugs in biological fluids. Table II shows that the low water solubility of sulfadiazine is increased 14 fold in M/15 phosphate buffer at pH 7.4. It is apparent that these data may account to a large extent for the differences in the absorption, distribution and excretion of these drugs. More detailed data on these points together with related data on the acetylated derivatives will be published elsewhere.[4] It also seems possible, however, that these data explain Marshall's observation[5] that sulfadiazine (ionized) seems confined to extracellular fluid whereas sulfanilamide (unionized) is distributed with body water.[6] Finally, utilization of

TABLE II.
Influence of Ionization on the Solubility of Sulfonamides.

	Solubility in H_2O 37°C	Solubility in M/15* phosphate buffer pH 7.4	No. of times increase in solubility
Sulfanilamide	1.400 mg per 100 cc	1,720 mg per 100 cc	1.2
Sulfapyridine	49.5 "	75 "	1.5
Sulfathiazole	94 "	248 "	2.6
Sulfadiazine	12.3 "	167 "	13.6

*All measurements were made after filtering off the excess solid phase of drug at 37°C. Drug concentrations were estimated by modifications of the method of Bratton and Marshall[10] using the Klett-Summerson photo-electric colorimeter. The pH of approximately 7.4 was obtained after the drugs were dissolved in buffers of higher pH.

[3] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[4] Jensen, O., and Fox, C. L., Jr., in preparation.

[5] Marshall, E. K., Jr., Conference on Chemotherapy, the New York State Department of Health, 1941.

[6] Painter, E., *Am. J. Phys.*, 1940, **129**, 744.

[10] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

this information has proved of value in providing new methods of local therapy with sulfonamide derivatives.[7]

It is a pleasure to thank Dr. David Rittenberg for suggestions with the physical chemical measurements, and Dr. Francis P. Chinard for his data on the dissociation constant of sulfanilamide.

13727

Mechanism of Sulfonamide Action. I. Acidic Dissociation and Antibacterial Effect.*

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The antibacterial efficiency of sulfonamides as measured by their ability to overcome *p*-aminobenzoic acid (PAB) has previously been shown to be dependent upon the pH of the medium.[1] On the other hand, since the relative efficiency of sulfanilamide and sulfathiazole is nearly constant at different pH values and independent of the bacterial species tested, the antibacterial property of each sulfonamide may be regarded as an inherent characteristic.[2]

The steric resemblance of sulfonamides and PAB and the lack of activity of the ortho and meta derivatives have been accepted as indicating a specific spatial charge distribution. In view of this specificity, one possible explanation of the difference in efficiency of the sulfonamides on the one hand, and of the significantly large ratios of sulfonamide to PAB on the other, might lie in wide variations in their acidic dissociation. This would lead to the conclusion that the effective constituent of sulfonamide solutions is either the anion or the zwitterion, rather than the undissociated acid or the cation.

Preliminary values of the acidic dissociation constants of sulfanilamide, sulfapyridine and sulfathiazole have been obtained using a

7 Fox, C. L., Jr., in press.

* The authors are grateful to Dr. William Mansfield Clark for advice on the mathematical interpretation of the data.

1 Schmelkes, F. C., and Wyss, Orville, *J. Bact.*, 1942, **43**, 71.

2 Wyss, Orville, Grubaugh, K. K., and Schmelkes, F. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 618.

TABLE I.
Acid Dissociation Constants of Sulfonamides.

Sulfanilamide	2.2×10^{-11}
Sulfapyridine	5.1×10^{-9}
Sulfathiazole	6.2×10^{-8}
p-aminobenzoic acid[3]	1.2×10^{-5}

solubility method. The constants calculated for the dissociation

$$NH_2RH \rightleftharpoons NH_2R^- + H^+$$

are listed in Table I.

The most widely accepted explanation of sulfonamide activity is based on its competition with PAB. On the hypothesis presented above it seemed reasonable to conclude that at a high pH, where a proportionately larger fraction of the dissolved sulfonamide exists in an anionic species, it will compete on a more favorable basis with *p*-aminobenzoate ion than at a low pH where acidic dissociation is relatively lower for the weaker acid. The following experimental data show that this is actually the case.

TABLE II.
Effect of $[H^+]$ upon Sulfonamide Bacteriostasis.

Inhibition of Growth of *E. coli* in a Synthetic Medium.			
pH	7.6	6.8	6.0
20 mg% sulfanilamide	95%	71%	54%
pH	6.7	5.7	4.6
5 mg% sulfadiazine	95%	89%	17%
Inhibition of Growth of *Strep. pyogenes* (1896 Lockwood) in Heart Infusion Broth.			
pH	7.6	7.1	6.7
Sulfanilamide mg%			
5%	40%	19%	0%
10%	49%	39%	8%

Using the dissociation constants it is possible to express the ratio of anion concentrations of two acids of different strength as a function of $[H^+]$, as follows:

$$\frac{C_B}{C_S} = \frac{[B^-]}{[S^-]} \left\{ \frac{K_S[H^+]}{([H^+] + K_S)K_B} + \frac{K_S}{[H^+] + K_S} \right\} \qquad (1)$$

where $[S]$ and $[B]$ denote the concentration of the sulfonamide and PAB anions, C_S and C_B the total concentrations and K_S and K_B the respective dissociation constants. When $[H^+]$ is at least 100 times as large as K_S then $([H^+] + K_S)$ may be simplified to $[H^+]$ thereby introducing an error not exceeding 1%. For such ranges of $[H^+]$ the equation can therefore be simplified to

$$\frac{C_B}{C_S} = \frac{[B^-]}{[S^-]} \left(\frac{K_S}{K_B} + K_S \cdot \frac{1}{[H^+]} \right) \qquad (2)$$

[3] Walker, *Z. physik. Chem.*, 1905, **51**, 709, and Holmberg, *Z. physik. Chem.*, 1908, **62**, 728.

The hypothesis that an anionic form is the effective agent requires that $[B^-]/[S]$ is a constant at any selected bactericidal effectiveness, for instance at one-half maximum growth. In $[H^+]$ ranges where Equation *2* applies, C_B/C_S plotted against $1/[H^+]$ should be a straight line. Also sulfonamides characterized by a higher K_S should give a plot with a steeper slope. On the other hand, as $[H^+]$ becomes significant with respect to K_S equation (1) applies. Accordingly the slope should decrease and become zero where dissociation is substantially complete.

Experimental. Bacterial growth rates were determined at a constant pH by using strongly buffered synthetic media and making measurements early in the log phase of growth where the hydrogen ion concentration was adequately controlled. The inoculated medium was divided into portions which were adjusted to the desired pH values with a few drops of concentrated acid or alkali. After a short incubation period the medium was dispensed into culture tubes containing a sulfonamide concentration well over the minimum required to inhibit growth and containing for each pH a series of PAB concentrations. Incubation was continued and growth was followed by turbidity measurements or plate counts. The growth rates were thus determined and the molar ratio of PAB to sulfonamide permitting a rate of growth one-half that of the control tube was computed for each pH. In the figure this molecular ratio C_B/C_S is plotted against the reciprocal of the hydrogen ion concentration.

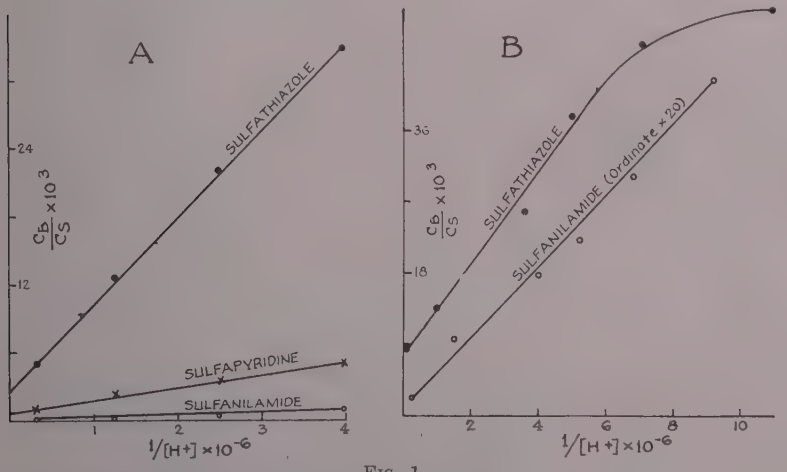

FIG. 1.

Mol ratio of PAB/sulfonamide permitting one-half maximum growth as an inverse function of the hydrogen ion concentration.

Values computed from growth studies on *E. coli* in synthetic medium at controlled pH.

From Fig. 1 A, it is apparent that in $[H^+]$ ranges where equation 2 applies, the predicted straight line plot is obtained, whereas in Fig. 1 B, it is shown that due to its high dissociation constant the sulfathiazole curve bends in accordance with equation 1. Moreover, the predicted relation between slope and K_S obtains.

As shown in Fig. 1 B and in accordance with equation 1 the increase of the efficiency of sulfathiazole with pH becomes progressively smaller, beginning approximately at pH 7. On the other hand, the corresponding point of inflection for sulfanilamide would be in the neighborhood of pH 10.5. If the pH is gradually increased above a value of 7, sulfanilamide should approach sulfathiazole in effectiveness. Preliminary experiments with organisms which grow in synthetic media buffered at slightly above pH 9 indicate that sulfanilamide actually approaches sulfathiazole in efficiency when this high pH is maintained.

Discussion. It should be borne in mind that while the degree of acidic dissociation governs the effectiveness of the active sulfonamides studied, other important factors are involved in the mechanism of sulfonamide action.

Further, this is only one aspect of the clinical problem which is complicated by considerations of absorption, solubility, toxicity, excretion, etc. Nevertheless, it would appear highly advantageous to maintain the pH of such infected loci that are to be treated with sulfonamides at the highest physiologically acceptable value. At pH 9 there is only comparatively little difference in the bactericidal effect of sulfanilamide and sulfathiazole.

Summary. From the nature of the function relating the effectiveness of sulfonamides to the hydrogen ion concentration the conclusion can be drawn that the active agent in a sulfonamide solution is an anionic species.

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Factors Influencing the Choice of Media for *in vitro* Sulfonamide Studies.

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Certain media are reported to inhibit the action of sulfonamides

in vitro. Lockwood[1] found that peptone inhibited the bacteriostatic and bactericidal action of sulfanilamide. MacLeod[2] has used a synthetic, inhibitor-free medium. A comparison of viable bacterial counts and bacteriostatic and bactericidal concentrations of sodium sulfathiazole in several of our media indicated that there was a direct relation between the nutritive quality of the media and the bacteriostatic and bactericidal concentrations of sodium sulfathiazole in the same media. In studying the growth curves of *Shigella paradysenteriae* Flexner in several media which we considered using for *in vitro* studies with sulfonamides, we observed (Table I) that certain media favored the development of a large bacterial population while other media supported the growth of only a small bacterial population. The synthetic medium[2] was composed of a mixture of salts with asparagine and glucose. This medium gave the least growth. Next in order were the nutrient broth, Bacto, which contained beef extract and peptone; and the 5% veal infusion, also a Bacto medium. The synthetic medium was much improved by the addition of 2% Witte's peptone. The brain-heart infusion medium Bacto, containing infusions from calf brains and beef heart together with proteose peptone and glucose, gave very much better growth than any of the others. Sodium sulfathiazole was added to these media in the following concentrations, 0.01 mg %, 0.1, 1.0, 5, 10, and increasing by 10 mg up to 100 mg %, then by 20 mg up to 200 mg %. When these media were inoculated with a few microörganisms we observed (Table I) that the medium which might be considered most inhibitory to the action of this sulfonamide was the medium which was most favorable to the development of the largest bacterial population, while the medium which appeared to be the least inhibitory was the one which was least favorable for an increase in population. In Table I are recorded the bacteriostatic and bactericidal concentrations of sodium sulfathiazole for *Shigella paradysenteriae* Flexner on 1, 2, 7, 14, and 21 days after inoculation of the various media with a small number of bacteria per cc. All media were incubated at 37°C for 4 weeks. Prolonged incubation resulted in increases in the bacteriostatic concentrations which in some instances occurred gradually and in others abruptly. When growth once appeared subcultures always remained positive for from 4 to 6 weeks. Viable bacterial counts during prolonged incubation in concentrations of sulfonamide which did not permit immediate growth showed that the original number of bacteria inoculated decreased

[1] Lockwood, J. S., and Lynch, Helen M., *J. A. M. A.*, 1940, **114**, 955.

[2] MacLeod, Colin M., *J. Exp. Med.*, 1940, **72**, 217.

TABLE I.

Effect of Quality of Medium, Bacterial Population and Time of Observation on Bacteriostatic and Bactericidal Concentrations of Sodium Sulfathiazole *in vitro*.

Media	Max.† viable counts‡ millions	Inocula per cc	Bacteriostatic conc. Days and mg % 1	2	7	14	21	Bactericidal conc. Days and mg % 1	2	7	14	21
Synthetic	10	46	*	*	*	*	*	*	*	*	*	*
Nutrient Broth	220	25	0.1	0.1	0.1	20	60	250	100	100	100	100
Veal Infusion	275	46	0.1	0.1	1.0	1.0	80	>150	150	80	80	80
Syn. + 2% Peptone	660	46	5.0	40	100	100	100	>400	400	100	100	100
Brain Heart Inf.	2080	25	<2.0	60	150	150	200	>200	>200	200	200	200

*No growth. Much larger inoculum necessary to obtain growth.
†Inoculum of 85 bacteria per cc in logarithmic phase.
‡Viable counts made after 12, 16, 20, 24, 30, 36 and 48 hours.

for a period of about 3 days and then increased, slowly at first, then rapidly, to a total number normal for that medium without sulfonamide. Growth became visible when the viable count was 15 to 20 million per cc.

Such late growth is suggestive of adaptation to environment. We have some data indicating that this may lead to the development of drug resistance *in vitro*. Such slow adaptation with its resultant increase in bacteriostatic concentration was observed in all media tested by us. The prolonged incubation would seem to introduce a factor which does not enter into the interesting report of Rose and Fox[3] which was published just as this paper was completed. They conclude that bacteria possess the ability to undergo only a certain limited number of cell divisions in the presence of a bacteriostatic concentration of a sulfonamide, regardless of the size of the inoculum. Their time of observation was only 4 days. Our results would indicate that a certain concentration of sulfonamide may be bacteriostatic for as long as 7 days (0.1 mg % in the case of nutrient broth, Table I) after which time growth occurred not only in the concentration which had been bacteriostatic for 7 days, but also in higher concentrations.

Frequent subcultures were made of each of the 19 different concentrations of drug to determine the bactericidal concentration of the sulfonamide in each medium. The bactericidal concentrations were high at first but decreased rapidly and usually within 7 days reached a level ultimately shown to be the maximum bacteriostatic concentration. It is observed in Table I that brain-heart infusion medium, which favored a high bacterial count, showed a bacteriostatic concentration on the second day of 60 mg % and on the 21st day 200 mg %. The bactericidal concentration was 200 mg %. Nutrient broth

[3] Rose, H. M., and Fox, Charles L., *Science*, 1942, **95**, 412.

which gave next to the lowest bacterial count showed a bacteriostatic concentration for the same microörganism on the second day of 0.1 mg %, and on the 21st day 60 mg %. The final bactericidal concentration in this medium was 100 mg %. It would seem, therefore, that values for the bacteriostatic and bactericidal concentrations of a sulfonamide compound for a certain microörganism *in vitro* are dependent upon certain variables such as nutritive quality of medium and time of observation.

A third factor influencing the bacteriostatic and bactericidal concentrations is the number of bacteria inoculated into the medium. The influence of this factor is demonstrated in Table II, where the inoculum for nutrient broth varied from 33 to 33,000 bacteria per cc. The results observed in Table II show that the bacteriostatic and bactericidal concentrations of sodium sulfathiazole and sulfaguanidine increased as the number of bacteria in the inocula were increased. For instance, the bacteriostatic concentration of sodium sulfathiazole was 1 mg % on the seventh day when the inoculum was 33 bacteria per cc, and 80 mg % on the same day with an inoculum of 33,000 bacteria per cc. The bactericidal concentration on the seventh day was 1 mg % with an inoculum of 33 bacteria per cc, and 100 mg % when the inoculum was 33,000 bacteria per cc. In general, the time of observation was again a factor as indicated by increasing bacteriostatic concentrations and decreasing bactericidal concentrations with continued incubation.

The same 3 factors exerted similar influences when sulfaguanidine was used but it is observed in Table II that this sulfonamide was much less bacteriostatic and bactericidal. The highest concen-

TABLE II.
Influence of Number of Bacteria in Inoculum.

Inocula *Shig. Para.* Flexner per cc	Sodium sulfathiazole in nutrient broth Days and mg%							
	Bacteriostatic conc.				Bactericidal conc.			
	2	7	14	21	2	7	14	21
33	1	1	1	1	10	1	1	1
330	1	1	1	10	20	10	10	10
3300	1	1	40	40	150	60	60	60
33000	1	80	80	100	200	100	100	100
	Sulfaguanidine in nutrient broth							
33	10	20	20	20	>200*	20	20	20
330	20	80	>200*	>200*	>200*	>200*	>200*	>200*
3300	40	>200*	>200*	>200*	>200*	>200*	>200*	>200*
33000	80	>200*	>200*	>200*	>200*	>200*	>200*	>200*

*Highest concentration used and not sufficient to give ultimate bacteriostatic and bactericidal effect with prolonged incubation.

tration of sulfaguanidine used in these experiments was 200 mg %, since solubility is only 220 mg %.

Summary. Studies of the effect of certain media on the action of sulfonamides indicate that a medium which may seem to be inhibitory is one which favors the growth and multiplication of bacteria so that an ultimate large bacterial population results. Our data also indicate that a medium, which by comparison may seem not to be inhibitory, is one which is not favorable to the development of large numbers of the bacteria under study. It is also apparent that the type of medium, time of incubation and number of bacteria inoculated are important factors which should influence conclusions drawn from sulfonamide studies *in vitro*. Prolonged incubation may result in adaptation of the bacteria to unusually high concentrations of the sulfonamide.

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Inhibition of Antimalarial Action of Sulfonamides by p-Aminobenzoic Acid.

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It has been found that p-aminobenzoic acid prevents the inhibitory action of sulfanilamide on the growth of hemolytic streptococci *in vitro*[1-3] and *in vivo*.[4] This compound also inhibits the therapeutic effect of sulfanilamide on mice inoculated intracerebrally with the virus of lymphogranuloma venereum.[4] It has been suggested[2, 4] that sulfanilamide exerts its action by competing for an enzyme associated with a metabolite similar in structure to p-aminobenzoic acid and essential to the organism.

Coggeshall[5] showed that sulfanilamide had a marked effect against plasmodia (*Plasmodium knowlesi* in rhesus monkeys). Other sulfonamides[6] were shown to be effective against *P. knowlesi* and against *Plasmodium cynomolgi* and *Plasmodium inui* in rhesus monkeys.

1 Woods, D. D., and Fildes, P., *Chem. and Ind.*, 1940, **59**, 133.
2 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.
3 Selbie, F. R., *Brit. J. Exp. Path.*, 1940, **21**, 90.
4 Findlay, G. M., *Brit. J. Exp. Path.*, 1940, **21**, 356.
5 Coggeshall, L. T., *Am. J. Trop. Med.*, 1938, **18**, 715.
6 Coggeshall, L. T., and Maier, J., *J. Inf. Dis.*, 1941, **69**, 108.

Sulfadiazine was shown[7] to be effective against human malaria (*Plasmodium vivax* and *Plasmodium falciparum*). The present paper reports the effect of p-aminobenzoic acid in inhibiting the action of sulfanilamide against plasmodia.

Plasmodium gallinaceum was used as the test infection in Leghorn or Rhode Island Red chicks weighing 50-100 g. Five birds were used for each test and 10 for each control group. The drugs used were mixed with a powdered chick mash so that the stated daily dose of drug was contained in 10 g of mash. This amount of drug-food mixture was consumed over a 24-hour period. Blood concentrations of sulfanilamide, determined by the method of Marshall and Litchfield[8] with a photoelectric colorimeter, were shown to be maintained at a uniform level over a 24-hour period by this method of administration. Drug feeding was continued for 10 days with blood level determinations on the 2nd and 3rd days. On the 2nd day 1,000,000 parasitized red cells suspended in 0.25 cc of sterile normal saline were injected intravenously. With this inoculum in untreated birds, parasites were first found in thin blood smears on the 3rd or 4th day. The parasite density increased rapidly until the 7th or 8th day, when 60 to 80% of the circulating erythrocytes were parasitized. Most birds died from the acute infection on the 8th to 12th day. Those which recovered usually died from the 19th to 23rd day with massive blocking of brain capillaries by exo-erythrocytic forms in capillary endothelial cells.

When known antimalarial drugs such as quinine and atebrin were tested in this manner, no parasites were found until several days after the termination of drug feeding. Following this the infection usually showed the same characteristics, including density and mortality, as those seen in untreated birds. Variations in these characteristics could be correlated with the size of the birds, because of rapid growth during the long incubation period, and not with the relative effectiveness of the drug. Accordingly, the length of the period from inoculation to first appearance of parasites was believed to represent the best criterion of effectiveness of a given drug. Table I shows the effect of quinine, atebrin, and sulfanilamide in increasing this interval.

In Table II are listed the results obtained when p-aminobenzoic acid was fed simultaneously with sulfanilamide, quinine, or atebrin. p-Aminobenzoic acid itself produced no increase in the incubation

[7] Coggeshall, L. T., Maier, J., and Best, C. A., *J. Am. Med. Assn.*, 1941, **117**, 1077.

[8] Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 85.

TABLE I.
Effect of Drugs on *P. gallinaceum.*

Drug	Dose mg/day/ chick	Dose mg/kilo/ day	Blood levels avg mg%	Day of first appearance of parasites (avg) Control group	Drug group	Effect
Quinine SO_4	10	130		3.0	13.4	+++
Atebrin HCl	8	100		3.0	16.8	+++
Sulfanilamide	20	400	3.3	3.2	12.2	+++
Sulfanilamide	40	800	7.0	3.2	17.0	+++

period and no change in the infection. At 2 dosage levels this compound inhibited completely the effect of sulfanilamide. In other experiments using 400 mg/kilo of sulfanilamide, the inhibitory effect was completely blocked by 0.25 mg/day of p-aminobenzoic acid and partially blocked by 0.1 mg/day, or a PABA sulfanilamide ratio of 1:400. Similar results were obtained with sulfathiazole and sulfadiazine. p-Aminobenzoic acid failed to inhibit the effect of quinine or atebrin.

These results suggest that the mechanism of inhibition by sulfonamides is similar for bacteria, viruses, and plasmodia. Quinine and atebrin apparently affect plasmodia through an entirely different mechanism than do sulfonamides.

TABLE II.
Effect of p-aminobenzoic Acid on Antiplasmodial Action of Drugs.

Drug	Dose mg/day/ chick	Dose mg/kilo/ day	Blood levels avg mg%	Day of first appearance of parasites (avg) Control group	Drug group	Effect on infection	Inhibition by p-aminobenzoic acid
p-aminobenzoic acid	25	300	3.1*	3.0	3.0	0	
sulfanilamide	20	400	3.3	3.2	12.2	+++	
sulfanilamide	25	300	9.0*	3.0	3.0	0	Complete
+ p-aminobenzoic acid	25	300					
sulfanilamide	25	400	4.8*	3.0	3.0	0	"
+ p-aminobenzoic acid	5	80					
quinine SO_4	8	160	12.2*	3.0	11.7	+++	0
+ p-aminobenzoic acid	40	800					
atebrin HCl	8	125		3.0	19.2	+++	0
+ p-aminobenzoic acid	25	400					

*Calculated as sulfanilamide.

13730

Morphologic Effects of Acute Inanition on Motor End Plates.*

Eben J. Carey.

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The significance of the pathologic effects of acute inanition of rats upon motor nerve plates is unknown. Sokoloff[1] stated that the nerve endings were enlarged in fasting summer frogs and Woollard,[2] that a similar change occurred in the motor nerve plates in the fasting albino rat. Jackson[3] demonstrated that the muscles lost weight almost 10 times faster than the morphologically resistant nervous system and that the spinal cord actually gained weight in his acute inanition series of the albino rats. He did not study the microscopic changes in the motor nerve plates nor the functional fibrillation of skeletal muscle following acute starvation. Speidel[4] observed the swelling of the growing sensory terminals in the tadpole after starvation. The purpose of this paper is to record the morphologic changes in the terminals of motor nerves following acute inanition in the albino rat.

Methods. Seventy well-nourished albino rats between 200 and 300 g had been fed oats, corn, meats and vegetables for 3 weeks before starvation began. They were kept in cages with wire net bottoms and allowed to drink freely of water. Four animals were killed with chloroform every 24 hours up to 15 days. Ten animals were used as controls. The modified gold method, Carey,[5] of teased intercostal muscle is preferred for demonstrating the continuity of the medullated nerve, end plate, and skeletal muscle fiber. The Bielchowsky silver and the intravitam methylene blue methods were used also. In 10-15-day starved, skinned rats under nembutal anesthesia (25 mg/kilo in 25% aqueous solution, injected intra-

* These investigations were carried out with the aid of grants for research to the Department of Anatomy of the Marquette University School of Medicine by the Committee on Scientific Research of the American Medical Association and the National Foundation for Infantile Paralysis, Inc.

1 Sokoloff, A. A., *Jahresb. d. Anat.*, 1876, **11**, 161.

2 Woollard, H. H., *J. Anat.*, 1927, **61**, 283.

3 Jackson, C. M., *Am. J. Anat.*, 1915, **18**, 75.

4 Speidel, C. C., *J. Comp. Neur.*, 1942, **76**, 57; *Proc. Am. Philo. Soc.*, 1942, **86**, 168.

5 Carey, E. J., *Anat. Rec.*, 1941, **81**, 393; Proc. Soc. Exp. Biol. and Med., 1941, **47**, 67; *Am. J. Path.*, 1942, **18**, 237.

peritoneally) functional fibrillation of intact, living muscle, characterized by slow spontaneous, incoördinated, ceaseless twitchings of 2 to 8 per second, was observed with a hand lens in reflected light and was studied with an oscillograph in the intact animal. The complete study of this functional fibrillation of skeletal muscle following acute inanition is reserved for a future communication.

Results. The normal motor end plates (Fig. 1), in 5000 measurements, varied from 24 to 55 microns in diameter, measured in the longitudinal direction of intercostal muscle fibers. During the first 72 hours of starvation the motor end plates (Fig. 2), in 3509 out of 5000 measurements, were enlarged and varied from 55 to 95 microns in diameter. During the first 72 hours of acute inanition the respirations in contrast to the normal of 30 to 60 per minute, varied from 30 to 100 per minute. Between the 4th and the 15th day there was a gradual decline in the respiratory rate to that of 10 to 40 per minute. The hyposarcolemmal axons underwent progressive retraction, loss of granular sole plate of Kühne, and an uprooting from the atrophic muscle fibers, from the 4th to 15th day of acute inanition. During the same period there was an enormous acute dilatation of the episarcolemmal axons and also of the exuberant axonic branches found between the muscle fibers (Fig. 3). The multiple branches of the episarcolemmal axons had periodic dilatations and constrictions. The dilatations formed large reservoirs, 20 to 40 microns in diameter (Figs. 4, 5, and 6). The axons of the motor nerves were evidently highly resistant to acute inanition and were stained deeply by gold chloride. The muscles, however, underwent progressive atrophy, close aggregation of cross striations alternating with those widely spaced, increased visibility of longitudinal striations, orientation of hyposarcolemmal nuclei, and finally degeneration of many fibers. From the 8th to the 15th day about 20% of the motor nerves ended freely in bulbous expansions found between the muscle fibers, which expansions were devoid of hypolemmal ramifications within the muscle fiber. This morphologic evidence pointed to a progressive denervation or uprooting of motor nerves in skeletal muscle following acute inanition. Differential fiber types due to fractional contraction in normal muscle (Fig. 1) became progressively less definite during inanition atrophy (Fig. 3). This confirmed the observations and reviews of Cobb,[6] Needham,[7] Hines,[8]

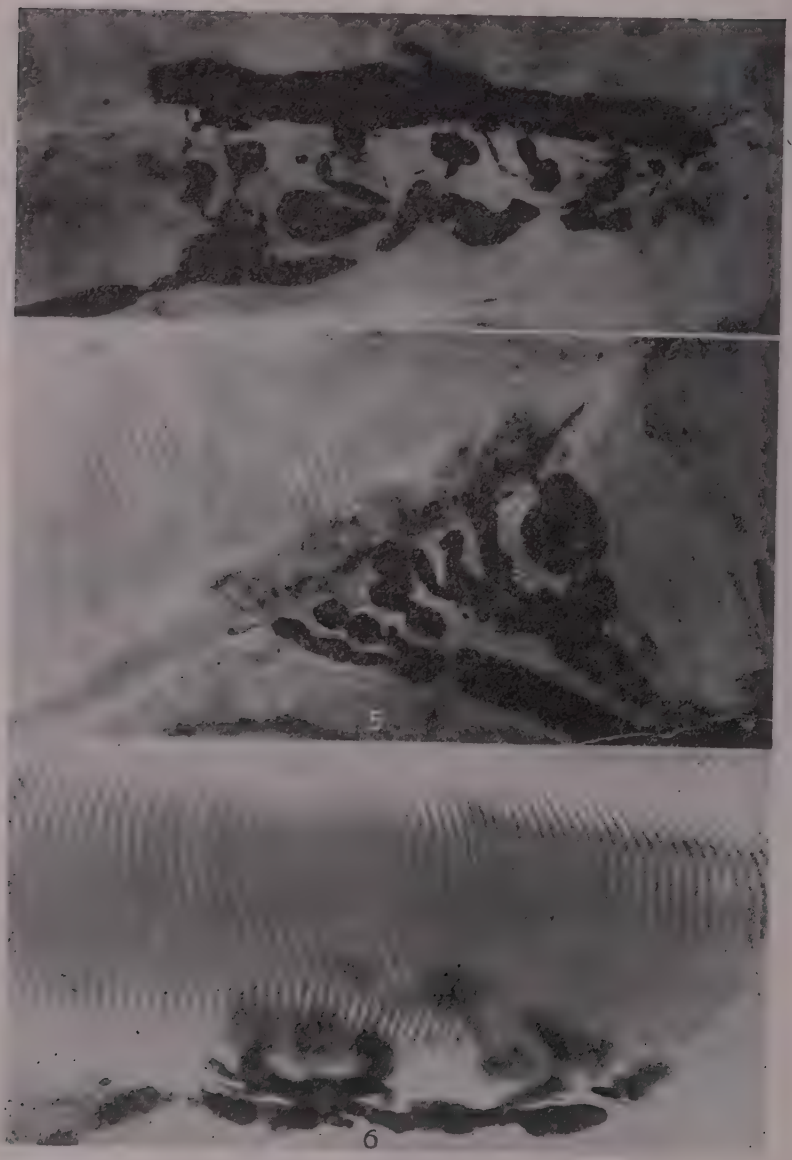
[6] Cobb, S., *Physiol. Rev.*, 1925, **5**, 518.

[7] Needham, D. M., *Physiol. Rev.*, 1926, **6**, 1.

[8] Hines, M., *Quart. Rev. Biol.*, 1927, **2**, 149.

FIGS 1, 2 AND 3.

Photomicrographs of teased normal intercostal muscle fibers and end plates of the white rat, Fig. 1; 72 hours inanition, showing enlargement of the end plates, Fig. 2; 8 days inanition, Fig. 3, showing dilated epilemmal axons outside of muscle fibers and different degrees of retraction of hypolemmal terminals of end plates within muscle fibers (gold chloride, × 150).



FIGS. 4, 5 AND 6.

Photomicrographs of motor end plates, profile view, in intercostal muscle eight days acute inanition, showing various degrees of dilatation of the epilemmal axons external to muscle fibers and progressive retraction of the hypolemmal terminals of the end plate within the muscle fibers. The granular sole plate of Kühne undergoes progressive dissolution during acute inanition (gold chloride, × 750).

Denny-Brown,[9] Hinsey,[10] Hines and Knowlton,[11] Tower,[12] and many others.

Summary. Starvation of the albino rat is fatal within 8 to 15 days and produces a structural detachment of about 20% of the motor nerves in skeletal muscle, morphologically manifested by: (1) progressive retraction and eventual uprooting of the hypolemmal end plate from within the atrophic muscle fiber and loss of the granular sole plate of Kühne, and (2) enormous distention of the epilemmal axons which stain very intensely with gold chloride.

13731

Age Factor in Responsiveness of Pituitary and Adrenals to Folliculoids.

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Several early investigators reported hypophyseal and adrenal enlargement following treatment with impure folliculoid preparations and Hohlweg[1] showed for the pituitary and Selye, *et al.*,[2] for both pituitary and adrenals that even crystalline estrone elicits these effects in the rat. Selye, *et al.*,[2] expressed the view that the adrenal enlargement is due to an increased secretion of the corticotropic hormone of the pituitary since it could not be obtained after hypophysectomy. Adrenocortical enlargement is a characteristic symptom of the alarm reaction and hence can be elicited by any nonspecific damaging agent. Histologically the cortical enlargement caused by folliculoids resembles that which appears during the alarm reaction,[3] yet it can hardly be regarded merely as a response to nonspecific damage as it is elicited by comparatively small doses of folliculoids which are not very damaging.

The observation that folliculoids enlarge the hypophysis and ad-

9 Denny-Brown, D., *Proc. Roy. Soc. B*, 1929, **104**, 371.

10 Hinsey, J. C., *Physiol. Rev.*, 1934, **14**, 514.

11 Hines, H. M., and Knowlton, G. C., *J. Physiol.*, 1939, **128**, 97.

12 Tower, S. S., *Physiol. Rev.*, 1939, **19**, 1; *Arch. Neurol. Psychiat.*, 1939, **42**, 219; *J. Neurophysiol.*, 1941, **4**, 398.

1 Hohlweg, W., *Klin. Wschr.*, 1934, **13**, 92.

2 Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1377.

3 Selye, H., Harlow, C. M., and Collip, J. B., *Endokrinologie*, 1936, **18**, 81.

renal cortex has repeatedly been described.[4, 5, 6] The following experiment confirms this fact and shows the intensity of the changes produced in this manner. Twelve male albino rats weighing 105-130 g were castrated and immediately afterwards 6 of them were injected subcutaneously once daily with 200 γ of estradiol in 0.1 cc of peanut oil while the remaining 6 acted as controls. After 20 days of treatment all animals were sacrificed and their adrenals and pituitaries weighed after fixation in "Susa" solution. The adrenals in the controls averaged 36 mg (range: 31-41 mg) and the pituitaries 8.4 mg (range: 7.5-8.8 mg). In the estradiol injected group the adrenals weighed 54 mg (range: 51-66 mg) and the pituitaries 15.9 mg (range: 14.8-16.9 mg). Both the enlargement of the adrenals and that of the pituitaries proved statistically highly significant ($P < 0.01$).

Since this type of response is invariably observed in adult rats, we were surprised to note that in a recent experimental series, in which immature rats were used, even very high doses of estradiol failed to cause pituitary or adrenal enlargement, indeed, they appeared to induce adrenal involution. Perusal of the literature indicates that only Clausen and Freudenberger[7] obtained a similar inverse response to folliculoids. They made no comment upon this finding, which was in apparent contradiction to all previously observed facts, but their protocols indicate that they also used immature animals. We felt that the age factor may be of great importance in determining the reaction of the hypophysis and adrenals to folliculoids since this factor proved to play a prominent rôle in other hormone-conditioned reactions, *e. g.*, the response of the ovaries to gonadotropic hormones,[8] the response of the uterus to testosterone,[9, 10] and the response of the male accessory sex organs to testoid compounds.[11]

Ten immature male albino rats weighing 40-60 g were gonadectomized. Five of them received 1 mg of α-estradiol in 0.1 cc of peanut oil subcutaneously bidaily on 10 consecutive days. The remaining 5 animals acted as controls, receiving only 0.1 cc of peanut oil subcutaneously bidaily given during the same period. On the

4 Ellison, E. T., and Burch, J. C., *Endocrinology*, 1936, **20**, 746.

5 Diaz, J. T., Phelps, D., Ellison, E. T., and Burch, J., *Am. J. Physiol.*, 1938, **121**, 794.

6 Albert, S., *Endocrinology*, 1942, **30**, 454.

7 Clausen, F. W., and Freudenberger, C. B., *Endocrinology*, 1939, **25**, 585.

8 Selye, H., and Collip, J. B., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 647.

9 Selye, H., *Anat. Record*, 1940, **76**, 145.

10 Selye, H., *Endocrinology*, 1940, **27**, 657.

11 Selye, H., and Albert, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 361.

11th day all animals were killed and their adrenals and pituitaries weighed after fixation in "Susa" solution. The adrenals in the controls weighed 22 mg on the average (range: 18-25 mg) and the pituitaries 5 mg (range: 4-6 mg). In the estradiol-injected group the adrenals weighed 16 mg (range: 15-19 mg) and the pituitaries 4 mg (range: 3-5 mg). While the decrease in the size of the adrenals proved statistically significant ($P < 0.01$) the apparent decrease in pituitary weight was not statistically significant ($P = 0.1$). Histologically the decrease in adrenal weight proved to be due to atrophy of the cortical cells. A repetition of this experiment under similar experimental conditions, but adding 2 groups of mature animals, one control, one estradiol-treated, yielded essentially similar results for the immature males, namely, a statistically highly significant ($P < 0.01$) adrenal involution without any significant change in pituitary weight. In the adults, on the other hand, estradiol elicited the usual statistically significant adrenal and pituitary enlargement. It appears that the responsiveness to folliculoid compounds is fundamentally different in immature and adult animals. Even such enormous doses as 2 mg of estradiol daily fail to cause adrenal or pituitary enlargement in the prepubertal rat while in the adult they always cause hypertrophy of these organs. This difference in responsiveness is not dependent upon the presence of an immature or mature gonad as it is demonstrable even in castrates.

Summary. Unlike in the adult animal, estradiol in high doses causes no pituitary or adrenal cortical hypertrophy in the immature rat, in fact it tends to decrease the size of these organs. This difference in hormone responsiveness is not due to the presence of a mature or immature gonad respectively since it is demonstrable even in castrates.

The expenses of this investigation were defrayed through a grant received by the DesBergers-Bismol Company of Montreal, Canada. The authors are greatly indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey, for the estradiol used in these experiments.

13732

Permeability of Red Blood Cells to Ions.

R. B. Dean. (Introduced by W. O. Fenn.)

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Recent experiments with radioactive isotopes have demonstrated the permeability of red blood cells to cations. The results obtained by different experimenters are, however, not directly comparable with each other or with the permeability of other systems. Permeability and diffusion phenomena ought to be expressed in standard c g s units.[1] With the aid of published measurements on cells of the same species[2] and with certain assumptions, it is possible to evaluate permeabilities in terms of standard units. Following Rashevsky[1] one may define the permeability, h, of a membrane to a given solute (provided the membrane is the limiting factor in the rate of exchange of solute) as

$$h = \frac{Q}{S\ (C_1 - C_2)}$$

where Q is the rate at which the solute crosses the membrane in mols per second, S is the area of the membrane and $C_1 - C_2$ the difference in concentration of the diffusing substance between the two sides of the membrane in mols per cc.

The permeability constant, h, of a membrane is defined on the assumption that the rate of diffusion, Q, is the difference between the rate of exit which is proportional to C_1, and the rate of entry, which is proportional to C_2. In the red cell, however, Q, the net rate of ion change, is zero. The permeability factor, b,[3] measures the fraction of the ions inside the cell which cross the membrane each minute. We may assume, for example, that loss of potassium from the cell is entirely passive and may be regarded as a leak, while the uptake of potassium takes place by another path motivated by an unspecified mechanism. If so, the rate of loss, b, should be proportional to C_1 and not to $C_1 - C_2$. In some experiments, b is measured by the rate of uptake of radioactive potassium; however,

1 Rashevsky, N., and Landahl, H. D., *Cold Spring Harbor Symposia*, 1940, **8**, 9.

2 Ponder, E., *The Mammalian Red Cell and the Properties of Haemolytic Systems*, Berlin, 1934.

3 Dean, R. B., Noonan, T. R., Haege, L., and Fenn, W. O., *J. Gen. Physiol.*, 1941, **24**, 353.

the rate of active uptake equals the rate of passive loss, if there is no net change in total potassium, so conversion of b to permeability units still entails dividing b by C_1. In the case of sodium or anions where there is no total change, we can calculate different constants depending on whether we assume that inward or outward movement is passive. In this paper I have assumed that outward movement is passive. In cases where permeability is measured by a net change of cellular content, of course, $C_1 - C_2$ must be used in the calculation. The concentration C_1 is that of the free potassium in the free water. Since there is no general agreement as to the existence or magnitude of the bound fractions of potassium and water, I have assumed that all the potassium is dissolved in all of the cellular water.

To determine the diffusion constant, D, we have to know the thickness of the cell membrane. For rabbit cells this lies between 110 and 120 Å[4, 5] but it has not been measured for other cells. The effective thickness of the membrane may be as low as 20 Å, the thickness of a monolayer of lipoidal material.[6] For the purposes of this calculation I have taken the larger value. The diffusion constant, D, is given as $D = hd$, where d is the membrane thickness in cm.

TABLE I.
Data Used to Evaluate the Permeability Constants of Various Ions.

	in vivo				*in vitro*
	Rabbit K^+	Human K^+	Dog K^+	Dog Na^+	Ox HCO_3^-
Volume, μ^3; $cm^3 \times 10^{-12}$	60	83	57	57	48
Surface, μ^2; $cm^2 \times 10^{-8}$	110	163	96	96	75
Cv, milimolar; mols, $cm^{-3} \times 10^{-6}$	91	101	8.0	98	11.0*
Ci, milimolar; mols, $cm^{-3} \times 10^{-6}$	144	158	12.4	153	17.2*
d, Å; cm $\times 10^{-8}$	110	(110)	(110)	(110)	(110)
b, $sec^{-1} \times 10^{-5}$	1.1	1.2	3.0	6	500,000
Q, mols, $sec^{-1} \times 10^{-20}$	6.2	11	1.4	34	26,000
h, cm, $sec^{-1} \times 10^{-10}$	3.9	4.1	11	23	200,000
D, cm^2, $sec^{-1} \times 10^{-16}$	4.3	4.5	12	25	220,000

Cv—Concentration of ions in the total cell volume.

Ci—Concentration of ions in the cell water.

*These values for ox cells refer to differences in concentration between cells and plasma.

d—Thickness of cell membrane which has only been measured for rabbit cells. Other cell membranes are assumed to have the same thickness.

b—Fraction of the ions in each cell which leaves each second.

Q—Number of mols of ions in each cell which leaves each second.

h—Is the permeability of the cell membrane to the ions.

D—Permeability constant of the substance forming the membrane to the ions.

In experiments done *in vivo* the cells were centrifuged immediately after the blood was drawn from the animal so that practically all the diffusion occurred while the cells were still in the animal.

[4] Waugh, D., and Schmitt, F. O., *Cold Spring Harbor Symposia*, 1940, **8**, 233.

[5] Fricke, H., Palmer, E., and Ponder, E., *J. Cell. Comp. Physiol.*, 1939, **13**, 69.

[6] Dervichian, D. R., and Macheboeuf, F., *Comptes Rendus*, 1938, **206**, 1511.

Table I shows values of the various factors and calculated values of Q, h, and D for potassium in rabbit, dog, and human cells,[3, 7] and for sodium in dog cells,[8] all *in vivo*. Where measurements of the cells were not available, a reasonable estimate has been made by comparison with other data. These values are indicated by parentheses.

The diffusion of bicarbonate ion out of ox cells *in vitro* has been estimated by analytical methods.[9] These results are not strictly comparable as some of the resistance to diffusion certainly lies in the fluid of the cell. Nevertheless, the data have been evaluated as if all the resistance were in the membrane. The results are probably valid to within an order of magnitude.

The probable errors of the determinations of diffusion rates, b, are of the order of 20%. The other values are probably more accurate, except for the thickness of cells other than the rabbit. Therefore, the constants given here can not be trusted very closely. They should be accurate as to order of magnitude.

For comparison, Table II lists diffusion in bulk materials as well as the diffusion constants reported by Jacobs on cells *in vitro*[10] and converted to c g s units assuming a membrane thickness of 110 Å. It is obvious from Table II that the red cell membrane is extraordinarily impermeable to cations and is about 50,000 times more permeable to anions. If the assumptions underlying these calculations are valid as to order of magnitude, the red cell membrane is less permeable than most dense solids which indicates a very high degree of association between the molecules of its structure. Incidentally, all the equations developed by Jacobs and his school[11] for

TABLE II.
Diffusion constants D in cm^2 sec^{-1}.

KCl in water	1.6×10^{-5}
$ZnSO_4$ in water	1.4×10^{-6}
Starch in water	$8. \times 10^{-7}$
Zn in Cu	2.3×10^{-13}
HCO_3 in ox cells	2.2×10^{-11}
K in rabbit cells	3.9×10^{-16}
Urea in ox cells	2.0×10^{-10}*
Glycerol in ox cells	2.1×10^{-14}*

*These calculations are based on an assumed thickness of 110×10^{-8} cm for the ox red cell membrane.

7 Mullins, L. J., Noonan, T. R., Haege, L., and Fenn, W. O., *Am. J. Physiol.*, 1941, **135**, 93.

8 Cohn, W. E., and Cohn, E. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 445.

9 Dirken, M. N. J., and Mook, H. W., *J. Physiol.*, 1931, **73**, 349.

10 Jacobs, M. H., *J. Cell. Comp. Physiol.*, 1934, **4**, 161.

11 Jacobs, M. H., *Cold Spring Harbor Symposia*, 1940, **8**, 30.

the responses of a cell having a membrane impermeable to cations will be approximately true for a cell having a membrane 0.00002 times as permeable to cations as to anions. The rate of loss of potassium from cells where the uptake may have been stopped by a poison is slow enough to be undetectable in the initial lag period found by Davson[12] for the loss of potassium from cells poisoned by respiratory poisons. In his experiments all of the loss must be attributed to a changed permeability of the membrane. The question as to what puts potassium back in the cell against a diffusion gradient remains unanswered. However, the experiments of Danowski[13] and Harris[14] show that metabolism is necessary for the continued uptake of potassium by the cells of stored blood.

13733

Non-Permeability of Blood Clot to Sulfonamide Drugs in Presence of Increased Temperature.

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Favorable results have been recently reported[1, 2] following the use of induced fever associated with sulfonamide drugs in the treatment of subacute bacterial endocarditis. That sulfonamide drugs do not penetrate fibrin or blood clot to any significant degree has been reported by two separate groups of observers, using different *in vitro* methods.[3, 4] However, a third has found that penetration does take place.[5] It occurred to us that if a high body temperature increased the effectiveness of the sulfonamide drugs in subacute bacterial endocarditis, it could do so only by bringing the drug into contact with the organism within the vegetation; *i. e.*, by increasing the permeability of the vegetation to the drug. To determine this we studied

12 Davson, H., *J. Cell. and Comp. Physiol.*, 1941, **18**, 173.
13 Danowski, T. S., *J. Biol. Chem.*, 1941, **139**, 693.
14 Harris, J. E., *J. Biol. Chem.*, 1941, **141**, 579.
1 Solomon, H. A., *New York State J. Med.*, 1941, **41**, 45.
2 Bierman, W., and Baehr, G., *J. A. M. A.*, 1941, **116**, 292.
3 Duncan, C. N., and Faulkner, J. M., *Am. J. Med. Sci.*, 1940, **200**, 492.
4 Friedman, M., *Arch. Int. Med.*, 1941, **67**, 921.
5 Uhley, M. H., and Katz, L. N., *J. Infect. Dis.*, 1941, **68**, 291.

the permeability of blood clot to various sulfonamide drugs under conditions of high temperatures simulating that of induced pyrexia.

Procedure. Our procedure consisted of suspending whole blood clot in serum containing a chemotherapeutic agent, following to some extent the method of Duncan and Faulkner.[3] Five cubic centimeters of human blood were withdrawn and allowed to clot and stand at room temperature for 24 hours. Two cc of a 25 mg % solution of a sulfonamide were then added to the serum surrounding the blood clot, and the whole allowed to stand for another 24 hours at room temperature (about 21°C). A similar preparation was kept for 24 hours at 37.5°C and a third at 41°C. At the end of this period, the serum was removed and the clot thoroughly washed and dried. The clot was then crushed in a mortar, boiled in water and filtered. Determinations were then made on the filtrate and previously removed serum of the sulfonamide level, using the method of Marshall and Bratton. The clot filtrate was prepared without boiling in a control experiment, to eliminate any possible destructive effects on the drug by boiling, with no difference in results.

The drugs used were sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, the sodium salts of sulfapyridine and sulfadiazine, and promin.

To determine whether heparin has any effect on the permeability of blood clot to the sulfonamide drugs, 10 units were added to 5 cc of clot and serum, which had been standing at room temperature for 24 hours. Experiments were then performed as above, using different drugs and conditions of temperature. The heparin content

TABLE I.
Concentrations of Various Sulfonamides in Blood Clot and Surrounding Serum under Different Conditions of Temperature.

	Concentration of drug in mg per 100 cc of solution					
Temperature:	21°C (approx.)		37.5°C		41.0°C	
Drug	Serum	Clot	Serum	Clot	Serum	Clot
Sulfanilamide	8.5	T*	10	0	9	T
Sulfapyridine	14.0	0	15	0	13	0
Sulfathiazole	11.0	0	11	0	12	0
Sulfadiazine	9.5	0	9	0	9	0
Sodium Sulfapyridine	—	—	—	—	17.5	T
Sodium Sulfadiazine	—	—	—	—	9	T
Promin	—	—	—	—	4	T
With Heparin added:						
Sulfanilamide	10.0	T	8	T	8	0
Sulfapyridine	15.0	T	13	T	17	T
Sulfathiazole	13.0	0	17	0	15	0
Sulfadiazine	12.0	0	16	0	14	0

*T—Trace.

of each tube was the equivalent of 10,000 units per 5000 cc of blood, which will produce *in vivo* a prolongation of coagulation lasting for about 4 hours.

Results. Table I shows the significant results. Repeat experiments were very closely confirmatory. Readings under 1.0 mg per 100 cc were called trace, a fractional reading being too inaccurate. As can be seen from the table, the amount of penetration of the blood clot under all conditions and with the different drugs was practically negligible. Heparin did not have any effect on the permeability of the blood clot.

Summary. The permeability of human blood clot to various sulfonamide drugs under conditions of increased temperature was determined in an *in vitro* study. Negligible penetration of the clot occurred even in the presence of temperatures up to 41°C. Heparin had no effect on the permeability of the blood clot.

13734

Evidence for the Existence of Two Antibodies for Crystalline Insulin.

FRANCIS C. LOWELL. (Introduced by Sanford B. Hooker.)

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Marked insulin resistance was discovered in a 43-year-old female diabetic exhibiting severe urticaria following injections of crystalline and regular insulin. The patient was studied as follows: (1) Intravenous insulin-tolerance tests were carried out with commercial crystalline insulin and with a preparation of human insulin; (2) skin tests and passive-transfer tests were made; (3) the patient's serum was tested for the presence of an insulin-neutralizing antibody by mouse-assay.

This patient has been reported previously as a case of allergy to insulin.[1]

Materials and Methods. Serum from the patient was sterilized by filtration through a Seitz filter and stored at 4°C.

Lilly's u40 commercial solution of zinc-insulin crystals derived from beef and pork pancreas was used in all tests except as mentioned below and will be referred to as crystalline insulin.

[1] Yasuna, E., *J. Allergy*, 1940-41, **12**, 295.

Human insulin was prepared from human pancreatic tissue obtained at necropsy 3 to 12 hours postmortem. Acid-extraction was done as described by Somogyi, *et al.*,[2] and the final product contained 7 units per cc.

Assay of insulin was carried out in starved mice.[3] Each animal was injected intraäbdominally with 0.5 cc of a dilution of insulin, and then all the animals were placed together in a room maintained at 38° C. The number of mice showing symptoms of hypoglycemia at the end of 90 minutes was noted. A dose of 0.02 units caused hypoglycemic convulsions or coma in 90-100% of mice.

Tests for insulin-neutralizing antibody were made by mouse-assay by injecting a mixture of the patient's serum and insulin in a total volume of 0.5 cc. In these experiments control tests were also made using a normal human serum. There are two theoretical objections to these tests. The patient's blood sugar was elevated and the increased glucose present in the serum might have masked the physiological effect of the insulin. This appeared unlikely as the amount of glucose present was sufficient to cover only about 0.001 units of insulin when undiluted serum was used. A second objection was the possibility that normal human serum contained sufficient insulin to increase the incidence of hypoglycemic symptoms in mice receiving normal serum. However, tests in which mice received insulin contained in 0.5 cc of saline and 0.5 cc of undiluted normal serum showed that normal serum did not increase the incidence of symptoms but, on the contrary, delayed the onset of symptoms slightly.

Intravenous insulin-tolerance tests were carried out with the patient in the fasting state. Three weeks before the tests were done, the patient was desensitized with carefully graded doses of insulin. Subsequently she received daily doses of 40 to 230 units of insulin. At the time the tests were done, the relatively large doses of insulin were having no observable effect on the diabetic state. The patient was hospitalized for the entire period of observation and received a diet of 1400 calories. No adrenalin or glucose was given for reactions occurring during the tests.

Passive-transfer tests with the patient's serum were done in subjects who were satisfactory recipients in that they exhibited no local reaction to the endermal injection of insulin diluted 1:10. Normal skin sites were injected with 0.1 cc of serum, diluted and undiluted, and tested 24 to 48 hours later with 0.02 cc of a 1:10 dilu-

[2] Somogyi, M., Doisy, E. A., and Shaffer, P. A., *J. Biol. Chem.*, 1924, **60**, 31.

[3] *The Biological Standardization of Insulin*, C. H. 398, League of Nations Health Organization, Geneva, April, 1926.

tion of crystalline insulin. The appearance within 10 to 20 minutes of a definite wheal and erythema, with or without itching, indicated the presence of skin-sensitizing antibody in the injected serum. Mixtures of 0.05 cc of the patient's serum and 0.05 cc of various dilutions of crystalline insulin were also injected into normal skin sites which were subsequently tested for passive sensitization with injections of insulin diluted 1:10. The amount of insulin necessary to destroy the ability of the patient's serum to confer sensitivity on normal skin was determined in this way.

Results. Intravenous Insulin-tolerance Tests. The patient's fasting blood sugar taken on numerous occasions varied between 330 and 380 mg 100 cc. A single intravenous injection of 30 units of crystalline insulin caused a slight rise in the blood sugar followed by a fall to a point slightly below the fasting level. There was no reaction to this injection.

On the following day, 30 units of human insulin were injected intravenously. A marked allergic reaction occurred within 15 minutes characterized by severe generalized urticaria and a feeling of constriction in the throat. It was identical with previous allergic reactions following injection of crystalline or regular insulin. After the urticaria subsided the patient felt well until about 90 minutes after the test was started. She then began to feel shaky and weak. A blood sugar done at this time was 59 mg 100 cc. Two days later, in order to show that the patient was still resistant to crystalline insulin, 120 units were given subcutaneously over a period of 8 hours. At the end of this time, the blood sugar was 395 mg 100 cc, somewhat higher than the usual fasting level.

The two insulin-tolerance tests are illustrated in the chart.

Tests for Insulin-neutralizing Antibody. Five mice were injected with 0.5 cc of the patient's serum to which had been added 0.02 units of crystalline insulin. No mice in this group showed any symptoms in 90 minutes. Four of 5 mice in a control test developed convulsions, and the fifth developed mild symptoms. In a second test, 8 mice were injected with 0.5 cc of the patient's serum diluted 1:10 to which were added 0.04 units of crystalline insulin. In this group, only 4 mice (50%) showed symptoms. This dose of insulin was twice the convulsive dose. In the control test with 5 mice, using normal serum instead of the patient's serum, all developed convulsions within 40 minutes. No insulin-neutralizing effect was demonstrable when human instead of crystalline insulin was used in mouse-tests. When the skin-sensitizing antibody in the patient's serum was destroyed by heating to 57°C for 2 hours, the neutralizing

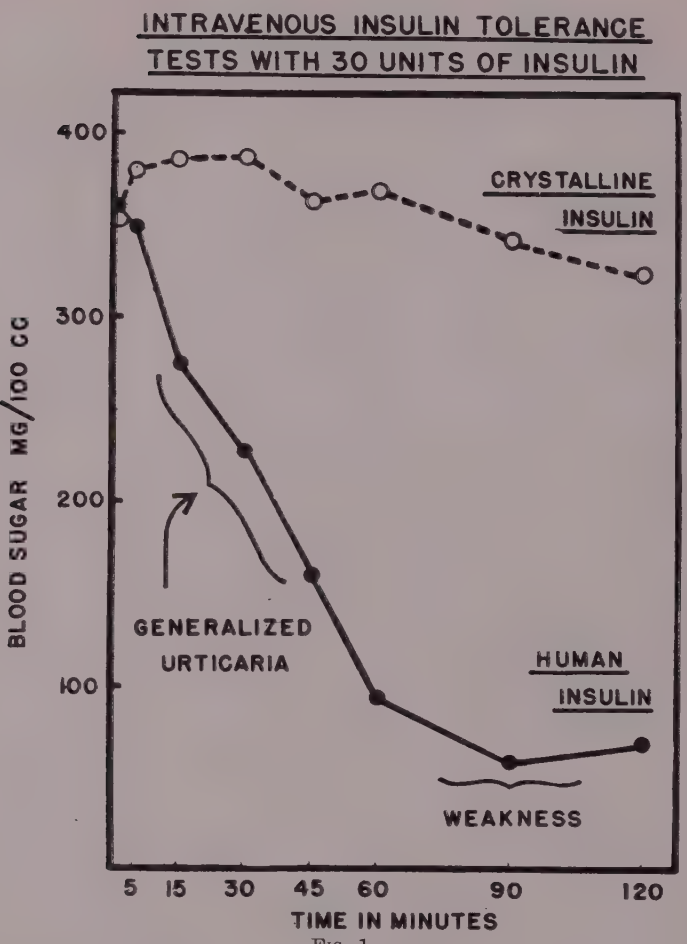

FIG. 1.

activity for crystalline insulin was still clearly demonstrable by the mouse-test.

Passive-transfer Tests. Skin-sensitizing antibodies were consistently demonstrable in all of 10 specimens of serum obtained from the patient over a period of 6 months. Sensitization of normal skin sites could still be obtained with most of these sera when they were diluted 1:16, 1:32, and, in some tests, 1:64. Mixtures containing 0.05 cc of serum and 0.04 units of crystalline insulin made up to a volume of 0.1 cc, were capable of sensitizing normal skin, but the addition of 0.4 units to 0.05 cc of serum destroyed the skin-sensitizing activity. The skin-sensitizing activity of the serum was also destroyed when

the serum was heated to 57°C for 2 hours. Skin-sensitizing antibodies were demonstrable in unheated serum when human, instead of crystalline, insulin was used for testing the sensitized sites.

Discussion. Allergy to insulin in the absence of resistance has been noted clinically, as well as resistance to insulin in the absence of allergy. Thus the two conditions can exist independently of each other. The data obtained from this patient are consistent with this and establish the following points: the patient was resistant to crystalline insulin but not to human insulin; the patient reacted allergically to both crystalline and human insulin and skin-sensitizing antibody was demonstrable for both insulins; the patient's serum contained an antibody for insulin, which protected mice from a lethal dose of crystalline insulin but failed to protect against human insulin; heating the patient's serum destroyed the skin-sensitizing antibody without destroying the insulin-neutralizing antibody. These findings indicate that there were two antibodies present in the patient's serum, an allergic antibody which was heat-labile and conferred sensitivity on normal skin, and an insulin-neutralizing antibody which was heat-stable and was capable of destroying the physiological effect of crystalline insulin. The presence of two antibodies for ragweed extract has been demonstrated in the serum of treated patients who are sensitive to ragweed by Loveless.[4] Two rough calculations can be made of the amount of the insulin-neutralizing antibody contained in the patient's total plasma volume, assuming this to be 2500 cc. In terms of insulin neutralized the figure is more than 2000 units by the endermal tests involving antibody-neutralization, and 1300 by the mouse-tests. From the quantitative point of view, both tests were crude, but the fact that the two figures are of the same order of magnitude suggests that the same antibody was active in both tests. It appears probable that the insulin-neutralizing antibody accounted for the patient's resistance to crystalline insulin. However, this antibody was not, strictly speaking, an antihormone because the patient responded well to human insulin. This suggests that the part of the molecule which served as antigen for the neutralizing antibody was characteristic for insulin derived from beef and pork pancreas and was not the part of the molecule which acted physiologically as insulin. The patient could perhaps be successfully treated if an insulin immunologically unrelated to beef and pork insulin were used. Human insulin was not available in quantities sufficient for treatment. The fact that the patient responded allergically to both crystalline and human insulin indicates

[4] Loveless, M. H., *J. Immunol.*, 1940, **38**, 25.

that the allergic antibody was alone responsible for the patient's skin sensitivity, generalized urticaria and other allergic symptoms, and was entirely unrelated to the patient's resistance to insulin.

Conclusions. Evidence is presented indicating the presence of two antibodies for crystalline insulin in the serum of a patient who was both allergic and resistant to crystalline insulin. It was also shown that the patient was allergic but not resistant to a preparation of human insulin. The relation of the two antibodies to the patient's response to crystalline and human insulin is discussed.

13735

Collodion-Agglutination Test in Human Tuberculosis.

RICHARD P. MORRIS. (Introduced by P. R. Cannon.)

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In human tuberculosis the precipitin-reaction, even utilizing a highly purified and reactive antigen, has been found positive in only about 12% of cases.[1] It was thought that the collodion-agglutination technic[2] would demonstrate precipitins in a much higher percentage of tuberculous humans. Previous reports concerning this latter method in human and experimental tuberculosis had suggested its accuracy and extreme delicacy.[3,4]

The technic as outlined by Cannon and Marshall[2] was rigidly adhered to in all details. As antigens, Old Tuberculin and a purified tuberculoprotein were used. The protein had been previously shown to be a superior antigen, *in vitro*, to Old Tuberculin.[1] For adsorption, 1 cc of OT in 19 cc of water was allowed to stand overnight with 20 cc of a dense suspension of collodion particles. For adsorption of the TPT, 20 mg of this was substituted for the 1 cc of OT, and the pH of the solution was adjusted to 5.8 with sodium-acetate acetic-acid buffer.

Over a period of several months, coated particles prepared in this manner were repeatedly tested with tuberculous human and rabbit sera, and normal human and rabbit sera. Some undoubtedly true and specific reactions were obtained in the animal sera, better reactions

1 Seibert, F. B., *Am. Rev. Tuberculosis*, 1930, **21**, 370.

2 Cannon, P. R., and Marshall, C. E., *J. Immun.*, 1940, **38**, 365.

3 Weir, J. M., personal communication.

4 Weir, J. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 47.

being obtained with TPT-coated particles than the OT-coated ones. However, the data could not be considered significant since many false reactions were also obtained in both animal and human sera. The unsatisfactory reactions were due to several causes, *viz.*, the use of suspensions of particles older than 24 hours, the use of non-inactivated sera, or sera more than 24 hours old, and to the varying effects of different speeds of centrifugation, time of incubation, etc. In other words, the collodion particles, particularly when mixed with sera in low dilutions, are liable to become flocculated because of the action of a variety of non-specific elements. It is for this reason, therefore, that the system must be rigidly controlled at all stages.

Fewer false positive reactions were obtained when fresh serum and freshly made particles were used. To enhance adsorption the tuberculoprotein was adjusted to a pH close to its isoelectric point before it was mixed with the suspension of particles. Even with these particles, titers below 1:60 were not considered reliable.

When these precautions were observed, it could be readily demonstrated that the coated particles were specifically agglutinated in high dilutions (1:3000) by antiserum from a goat immunized with dead tubercle bacilli. The OT-coated particles, however, when tested against 8 sera from tuberculous patients of different types gave no positive reactions. The TPT-coated particles, when tested against 24 sera from tuberculous patients of all types showed only one probably true reaction (titer 1:128) and 5 doubtful reactions below 1:60. Nor could positive reactions be obtained with either type of particle with sera from humans with positive cutaneous reactions but no active tuberculosis.

Conclusion. After several of the causes for false positive reactions in this method were eliminated, about 4% of human tuberculous sera gave specific positive reactions.

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Two New *Salmonella* Types with Undescribed Flagellar Antigens.*

D. W. Bruner and P. R. Edwards.

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A. *Salmonella simsbury.* The only culture of *Salmonella simsbury* encountered was received from Mr. E. K. Borman, who identified the organism as a *Salmonella* and sent it to writers for antigenic analysis. The bacillus was isolated from the feces of a normal human carrier. On subsequent examination no *Salmonella* could be isolated from this individual.

The organism was a motile bacillus which possessed the morphological, cultural and biochemical characteristics generally attributed to the genus *Salmonella*. Acid and gas were produced from glucose, arabinose, xylose, rhamnose, maltose, trehalose, dulcitol and sorbitol. Lactose, sucrose, inositol, adonitol and salicin were not attacked. Dextro-tartrate, levo-tartrate, mucate and citrate were fermented but meso-tartrate was not utilized. The organism produced hydrogen sulphide but did not form indol nor liquefy gelatin.

Alcohol-treated suspensions of the organism were agglutinated strongly by *S. senftenberg* (I, III, XIX) serum and to a lesser degree by *S. anatum* (III, X, XXVI) and *S. newington* (III, XV) serums. When used as antigen in absorption tests, *S. simsbury* removed all somatic agglutinins from *S. senftenberg* serum. *S. simsbury* was then used to prepare an agglutinating serum and it was found that all somatic agglutinins were removed from the serum when it was absorbed with *S. senftenberg*. The somatic antigens of *S. senftenberg* and *S. simsbury* are identical and are represented by the formula I, III, XIX.

When a formalized broth culture of *S. simsbury* was tested with serums derived from all the known flagellar antigens of the *Salmonella* group, it was flocculated by none of them. Likewise, *S. simsbury* serum which had a flagellar titer of 1 to 20,000 for the homologous strain, failed to flocculate any of the known flagellar antigens in a dilution of 1 to 100. It is apparent that the flagellar antigens of *S. simsbury* are not related to any of those hitherto de-

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

scribed and to them is assigned a new symbol, z_{27}. The antigenic formula of *S. simsbury* is I, III, XIX : z_{27}.

B. *Salmonella tennessee*. The sole representative of this type was received from Dr. W. C. Williams. It was isolated in the laboratories of the Tennessee Department of Health from the feces of a normal carrier who was employed as a food handler in a fraternity house, a number of the occupants of which were affected with food poisoning. No paratyphoid bacilli were isolated from the cases of food poisoning. Except for prompt production of acid and gas from inositol by *S. tennessee*, its cultural and biochemical characteristics were the same as those given for *S. simsbury*.

On serologic examination alcohol-treated suspensions of the bacillus were agglutinated strongly by *S. thompson* (VI, VII) serum and to a lesser degree by *S. newport* (VI, VIII) serum. When tested with single-factor serums it was found to react both with VI_1 and VI_2 serums of Kauffmann[1] and with VII serum. It was unaffected by VIII serum. When used as antigen in absorption tests, *S. tennessee* removed all somatic agglutinins from *S. thompson* serum. Likewise, an agglutinating serum prepared from *S. tennessee* was exhausted of somatic agglutinins by absorption with *S. thompson*. The somatic antigens of *S. tennessee* are VI, VII.

The flagellar antigen of *S. tennessee* had little serologic relationship to those of any of the known *Salmonella* types. Formalized broth cultures of the organism were not flocculated by any of the serums representative of the recognized flagellar antigens. An agglutinating serum derived from *S. tennessee*, which had a flagellar titer of 1 to 40,000 for the homologous strain flocculated the specific phase of *S. glostrup* (z_{10}) in a dilution of 1 to 400. It failed to agglutinate any of the other known *Salmonella* antigens in a dilution of 1 to 100. To the flagellar antigen of *S. tennessee* a new symbol, z_{29}, is assigned. The antigenic formula of the organism is VI, VII : z_{29}.

Summary. Two new Salmonella types with undescribed flagellar antigens were reported. *Salmonella tennessee* was represented by the antigenic formula, VI, VII : z_{29}. The antigens of *Salmonella simsbury* were characterized as I, III, XIX : z_{27}. Each type is represented by a single strain isolated from the feces of normal human carriers.

[1] Kauffmann, *Z. f. Hyg. u. Infektionskr.*, 1937, **120**, 177.

13737

Cure of Experimental Staphylococcal Meningitis.*

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Purulent meningitis was induced in rabbits, employing a technical procedure essentially the same as that of Felton[1] and Wegeforth, by intracisternal injection of *Staphylococcus aureus,* using cultures of a strain (Sca) derived from human meningitis and also cultures of the same strain after rabbit passage (237). Some of these animals were then subjected to treatment with bacteriophage, sulfathiazole or with both these agents.

Table I presents briefly the results in a preliminary group of 6 rabbits. The untreated controls died on the second day. The other 4 animals were treated with bacteriophage, beginning a few hours after inoculation. Only one survived, Rabbit 261, apparently the pioneer observation of recovery from experimental staphylococcal meningitis. The culture strain Sca, maintained on agar slants, gradually lost virulence so that not every control animal inoculated with a minute dose would die of meningitis.

The results in a group of 10 rabbits inoculated with strain 237 on March 31 are shown in Table II. The 4 untreated control animals all died of meningitis by the 5th day. The other 6 animals were treated

TABLE I.

Experiment 8. All rabbits were inoculated with 0.2 cc bacterial suspension, representing 11,000 living cocci of *Staphylococcus aureus,* Sca, into the cisterna magna, while under deep ether anesthesia on February 3, 1942. All these animals had positive cultures of fluid withdrawn by cisternal tap on February 5. Two controls (C) were untreated; the others were treated with bacteriophage. (I) inoculation; (T) treatment; (D) death; (L) lived.

	Feb. 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
225 C	I		D				hemorrhage; meningitis; culture positive											
260 C	I		D									,,		,,			,,	
228 T	I T	TD					hemorrhage;					,,		,,			,,	
227 T	I T	T	TD									,,		,,			,,	
226 T	I T	T	TD									,,		,,			,,	
																accidental death		
261 T	I T	T	T	T	T	T	T	T	T	T	T	T						L
											cisternal tap Feb. 18; culture negative							

* Aided in part by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry of the American Medical Association.

1 Weed, Lewis H., Wegeforth, Paul, Ayer, James B., and Felton, Lloyd D., *Monographs of the Rockefeller Institute for Medical Research,* No. 12, 1920, 5.

TABLE II.

Experiment 16. All rabbits were inoculated with 0.2 cc bacterial suspension representing 21,000 living cocci of *Staphylococcus aureus*, 237, into the cisterna magna, while under deep ether anesthesia on March 31, 1942. All these animals had positive cultures of fluid withdrawn by cisternal tap on April 1. Four controls (C) were untreated; the others were treated with sulfathiazole. (I) inoculation; (T) treatment; (D) death; (L) lived.

	Mar. 31	Apr. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
935 C	I	D							meningitis; culture positive								
941 C	I	D							,,			,,			,,		
944 C	I				D				,,			,,			,,		
993 C	I				D				,,			,,			,,		
930 T	I	T	TD						,,			,,			,,		
936 T	I	T	T	D					,,			,,			,,		
928 T	I	T	T	T	TD				,,			,,			,,		
969 T	I	T	T	T	T	D			,,			,,			,,		
965 T	I	T	T	T	T	T		D	,,			,,			,,		
959 T	I	T	T	T	T	T		negative cisternal fluid 4/14									L

with sulfathiazole beginning on the day of inoculation, and of these 5 died of meningitis and one survived.

Other experiments, in which this same culture strain was used and both bacteriophage and sulfathiazole were administered revealed a sharper contrast between treated and untreated animals.

Table III indicates briefly the result in one such experiment. All 4 of the untreated controls developed purulent meningitis and were dead on the 4th day. The 3 treated late with bacteriophage and sulfathiazole were likewise all dead on the 4th day. Of the 4 treated

TABLE III.

Experiment 13. All rabbits were inoculated with 0.2 cc bacterial suspension, representing 21,000 living cocci of *Staphylococcus aureus*, 237, into the cisterna magna, while under deep anesthesia, on March 10, 1942. All these animals had positive cultures of fluid withdrawn by cisternal tap on March 12. Four controls (C) were untreated; 4 were treated early with sulfathiazole and bacteriophage; 3 were treated late with the same agents. (I) inoculation; (T) treatment; (D) death; (L) lived. Cisternal fluids of rabbits 285, 286 and 287, taken March 23, were negative on culture.

	Mar. 10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
293 C	I			D						meningitis; culture positive									
292 C	I			D						,,					,,		,,		
294 C	I			D						,,					,,		,,		
295 C	I		D							during tap;					,,		,,		
289 T	I	T	D							meningitis;					,,		,,		
288 T	I	T	T	D						,,					,,		,,		
290 T	I	T	T	D						,,					,,		,,		
284 T	I	T	T	TD						during tap; hemorrhage; meningitis; culture positive									
285 T	I	T	T	T	T	T	T	T	T	T	T	T	—	cis-					L
286 T	I	T	T	T	T	T	T	T	T	T	T	T	—	ternal					L
287 T	I	T	T	T	T	T	T	T	T	T	T	T	—	fluids					L

within a few hours after inoculation, 1 died of hemorrhage from injury by cisternal tap, but the other 3 survived and on the 14th day of the experiment all 3 had negative cisternal fluids, sterile on culture. The protocol of one of these animals may serve to illustrate the rather strenuous therapeutic program.

Rabbit 286 was subjected to cisternal tap on March 10, with removal of 1 cc clear fluid and intracisternal injection of 0.2 cc bacterial suspension (21,000 living cocci). He was in good condition after the tap. After an interval of 4½ hours, treatment was begun and on this first day sodium sulfathiazole, 500 mg, was given by intravenous injection, sulfathiazole, 250 mg, intraperitoneally and bacteriophage, 100 cc, intravenously in 5 doses. On March 11, sodium sulfathiazole, 250 mg, was given intravenously, sulfathiazole, 250 mg, intraperitoneally and bacteriophage, 40 cc, in 2 intravenous doses. On March 12 there was generalized tremor. Cisternal tap yielded 0.2 cc bloody fluid containing many pus cells with rich growth of *Staphylococcus aureus* in 24 hours. The animal was distinctly weaker after the tap. Again sodium sulfathiazole, 750 mg, intravenously in 2 doses, and sulfathiazole, 250 mg, intraperitoneally, were given during the day as well as 100 cc bacteriophage in 5 intravenous doses. At the end of the day the rabbit was weaker, tense and jittery. On March 13 he appeared very weak but was again able to eat. Two intravenous injections of sodium sulfathiazole, each 250 mg, were given and also 2 intravenous doses of bacteriophage, each 20 cc. On March 14, sodium sulfathiazole, 250 mg, was given intravenously and 40 cc bacteriophage in 2 intravenous injections. The animal was much improved. For the next 3 days he received only one daily intravenous injection of 20 cc bacteriophage and on March 18, 19 and 20, only one daily injection of 10 cc, followed by an injection of 20 cc on March 21. After a free interval of 2 days a cisternal tap on March 23 yielded 0.2 cc clear fluid, the culture of which remained sterile. The animal has remained well, without sequelae.

Table IV summarizes the preliminary group of experiments with staphylococcal strain Sca. The later experiments in which the more satisfactory strain 237 was employed are summarized in Table V. The efficiency of the combined treatment with bacteriophage and sulfathiazole was very striking, especially when the animal survived the 3rd day.

Further studies will undoubtedly permit simplification of the therapeutic program and will probably permit a larger proportion of recoveries. Intrathecal injection of the therapeutic agents has shown

TABLE IV.
Summary of Experimental Staphylococcal Meningitis in 49 Rabbits Inoculated by Cisternal Puncture with Bacterial Strain Sea.

	Day of death											Meningitis	
	1	2	3	4	5	6	7	8	9	10	Later	Died	Lived
Untreated	—	1	4	4	1	—	—	1	—	—	2	13	4
Early Sulfathiazole	—	1	2	4	2	3	1	—	—	—	1	14	1
Early Bacteriophage	—	1	5	2	—	—	—	—	—	—	—	8	3
Late Sulfathiazole	—	2	2	—	—	—	—	—	—	—	—	4	0
Late Bacteriophage	—	—	1	1	—	—	—	—	—	—	—	2	0

TABLE V.
Summary of Experimental Staphylococcal Meningitis in 56 Rabbits Inoculated by Cisternal Puncture with Bacterial Strain 237.

	Day of death											Meningitis	
	1	2	3	4	5	6	7	8	9	10	Later	Died	Lived
Untreated	—	7	6	5	2	—	3	—	—	—	—	23	0
Early Sulfathiazole	—	1	1	1	1	—	—	1	—	—	—	5	1
Early Sulfathiazole and Bacteriophage	1	7	5	—	—	—	—	—	—	—	—	13	9
Late Sulfathiazole and Bacteriophage	—	1	1	2	1	—	—	—	—	—	—	5	0

promising results, in spite of the risk of death by trauma in the presence of increased vascularity incident to the disease in animals with such small cisternal spaces. The timing of the doses and the sympathetic care of the animals has seemed important.

These observations are in accord with our knowledge of human staphylococcal meningitis,[2] which, until recently, has been almost invariably fatal.

[2] MacNeal, Ward J., *N. Y. State J. Med.*, 1941, **41**, 1531; Weary, Willard B., and Lyons, John J. A., *N. Y. State J. Med.*, 1941, **41**, 2124; MacNeal, Perry S., and Foster, D. Bernard, *Am. J. Med. Sci.*, 1941, **202**, 874.

13738

Detection of Antibody by Complement-Fixation in Sera of Man and Monkey Convalescent from Mumps.*

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The importance of mumps as a disease among military personnel is considerable. In the last war it stood third among the diseases causing time lost from duty, being surpassed only by venereal diseases and influenza.[1, 2]

Although the work of Johnson and Goodpasture,[3] using *Macaccus mulatta* as the experimental animal, established the etiological agent of mumps as a filterable virus, the practical implications of their findings have not been realized. Thus, no laboratory tests for the diagnosis of the infection or tests for susceptibility are available. Moreover, no means are at hand by which the potency of convalescent sera which have been employed as prophylaxis may be assayed. Accordingly, we undertook the repetition of the basic experiments of Johnson and Goodpasture with the object of extending them in these directions. In this paper we report briefly on a complement-fixation test for the detection and estimation of antibody in the sera of man and monkey convalescent from mumps.

About 3 cc of saliva collected on the 1st and 2nd days of the disease from a typical case of epidemic parotitis was introduced into Stensen's duct of a monkey (*Macaccus mulatta*). On the 5th day the gland, which was somewhat enlarged, was removed under ether anesthesia, weighed and emulsified by grinding in infusion broth to make a 20% suspension. This was then inoculated into both ducts of another monkey. The animal, on the 7th day following inoculation, developed marked swelling of both parotid glands with considerable subcutaneous edema of the cheeks. In this way the virus has been passed in series through 5 monkeys—the last of

* This work in part has been carried out as one of the projects of the "Chemical, Clinical and Immunological Investigations of Human and Other Serum Globulins," under contract with the Committee on Medical Research of the Office of Scientific Research and Development.

[1] Michie, H. C., *The Medical Department of the United States Army in the World War*, Vol. IX, pp. 451-462, Government Printing Office, Washington, D.C., 1928.

[2] Wesselhoeft, C., *New England J. Med.*, 1942, **226**, 530.

[3] Johnson, C. D., and Goodpasture, E. W., *J. Exp. Med.*, 1934, **59**, 1.

which received material obtained by filtration of the parotid suspension through an Elford collodion membrane of 515 mμ pore diameter. The filtrate, which was shown to be bacteriologically sterile by aerobic and anaerobic incubation in suitable media, produced a disease in all respects similar to the unfiltered suspensions. Examination of sections of the parotid glands from all the infected monkeys revealed the pathological changes described by Johnson and Goodpasture.[4] Such changes were not observed in sections of glands removed from monkeys dying either of poliomyelitis or dysentery.

Antigens for use in complement-fixation tests were prepared by grinding with sand or alundum weighed portions of infected glands removed at the height of the disease. Sufficient physiological salt solution was added to make a 20% suspension. The suspension was then centrifuged at about 750 rpm for 10 minutes and the supernatant fluid was stored in sealed tubes in a CO_2 box (infected parotid suspension). Parotid glands removed from monkeys infected with dysentery bacilli or the virus of poliomyelitis were treated in the same manner to obtain suspensions of parotid to be used as controls (non-infected parotid suspension). At the time a test was set up, parotid suspensions were diluted with 5 times their volume of salt solution and centrifuged at 3500 rpm for 30 minutes in an angle centrifuge. Of the supernatant fluids, amounts of 0.2 cc were used in the tests.

The sera which have been tested for the presence of complement-fixing antibody have been obtained from monkeys before and at various times after infection with the virus of mumps and from human beings during acute stage of the disease and convalescence. To remove the anticomplementary activity which was occasionally marked, particularly in the monkey sera, the latter were heated at 62.5°C for 20 minutes. Satisfactory results were usually obtained with human sera which were heated at 60°C for 20 minutes. This procedure, suggested by Casals and Palacios,[5] was effective not only in removing the anticomplementary effect of sera, *per se*, but also in eliminating a certain amount of non-specific fixation which sometimes occurred with parotid suspension from monkeys not suffering from mumps. Sera were diluted by two-fold decrements and 0.25 cc of each dilution was employed in the test.

Pooled guinea pig serum was used as complement. This was preserved in the CO_2 box where its titer appeared to remain essentially unchanged for at least 2 weeks. Two units of complement contained in 0.25 cc were employed.

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4 Johnson, C. D., and Goodpasture, E. W., *Am. J. Path.*, 1936, **12**, 495.

5 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

The hemolytic system consisted of 0.5 cc of a 1% suspension of sheep red blood cells sensitized with 2 units of antisheep cell rabbit serum.

The test was carried out in the following manner: Diluted, inactivated serum, complement, antigen (parotid suspension from infected monkey) and sufficient saline to bring the volume to 1 cc were added in that order. The controls included in each test were as follows:

1. Complement alone
2. Serum alone
3. Serum and complement
4. Serum + complement + non-infected parotid
5. Infected parotid alone
6. Infected parotid + complement
7. Non-infected parotid alone
8. Non-infected parotid + complement
9. Salt solution

The mixtures were kept at 4°C for about 18 hours, when the sensitized cells were added. After incubation for ½ hour at 37°C in the water bath, the mixtures were again placed in the icebox and final readings made after the unhemolyzed cells had settled. In the tables dilutions of serum denote those which existed in the mixtures before the addition of sensitized cells.

The results so far obtained may be summarized as follows: The sera of monkeys taken before inoculation with the virus of mumps or at the height of the disease have not fixed complement in the presence of the suspension of infected parotid. Sera obtained from 6 monkeys during various periods of convalescence have, in every instance, shown various capacities specifically to fix complement. The titers, taken as "two-plus" fixation, have ranged between 1:128 and 1:2048. Complement-fixing antibodies persist in the sera of

TABLE I.

Complement-fixation Tests on Specimens of Serum from a Monkey Inoculated with the Virus of Mumps.

Time serum drawn Days after inoculation	Reciprocal of serum dilution							Control Non-infected parotid + serum 1:16
	16	32	64	128	256	512	1024	
0	0	0	0	0	nd	nd	nd	0
6†	tr	0	0	0	0	0	0	0
19	++++	++++	++++	++++	++++	+++	+++*	0
34	++++	++++	++++	++++	++++	+++	++	0

++++ = complete fixation
tr = trace of fixation
0 = complete hemolysis
nd = not done

*A subsequent titration of this specimen gave ++ fixation when diluted 1:2048.
†This specimen was taken during the height of the disease.

TABLE II.
Complement-fixation Test on Sera from Human Cases of Mumps.

Patient	Time serum drawn Days after onset	Reciprocal of serum dilution 8	16	32	64	128	256	512	1024	Control Non-infected parotid + serum 1:16
A	4	tr	0	0	0	0	0	0	nd	0
	17	tr*	++	+±	0	0	0	0	nd	0
	26	+++±	+++±	+++±	+±	tr	0	0	nd	0
O	6	+++±	+++±	+++	+±	0	0	0	nd	0
	24	++++	++++	++++	++++	+++	+	tr	nd	0
E	4	0*	±	±*	±	tr	0	0	nd	0
	23	++++	++++	+++	++±	++	+±	+	0	0

++++ = complete fixation.
tr = trace of fixation.
0 = complete hemolysis.
nd = not done.
*A suggestion of prozone occurred in these sera which gave very weak reactions.

monkeys for at least 3½ months following infection. The results of complement-fixation tests done on samples of serum obtained from one monkey at various times are presented in Table I.

The results of titrations carried out in the same manner on sera obtained from human beings during the acute and convalescent stages of mumps are presented in Table II.

It will be observed that whereas the specimen obtained from patient "A" during the active disease exhibited practically no fixation, slight evidence for the presence of antibody was obtained in the "acute" serum of patient "E" and definite indication of such a factor in that of the corresponding serum of patient "O". These findings strongly suggest that complement fixing antibody may appear early in certain individuals in contrast to others in whom its development may be delayed. The alternative hypothesis that antibody was present at the onset of the disease in the blood of patient "E" is manifestly improbable because of the weak reactions and relatively low titer of the acute serum compared with those of the specimen taken during convalescence. In the case of patient "O" this possibility definitely exists, but again is unlikely because of the rise in titer and increase in intensity of fixation shown by the specimen obtained on the 24th day. Certainty in respect to the question of whether or not complement-fixing antibody may sometimes be present at the onset of clinical symptoms must await the accumulation of additional data.

Although complement-fixing antibody is not always correlated with the protective capacity of antisera against viral agents, this is frequently the case. Its assay, then, in convalescent and normal human sera might serve as a tentative index to their value as pro

phylactic agents until a satisfactory technic for the evaluation of protective antibody becomes available.

The complement-fixation test should also prove useful in determining the presence or absence of virus in attempts to propagate the latter in tissue cultures, the developing chick embryo or in material obtained from animals hitherto regarded as insusceptible.

Finally, it is not impossible that when appropriate surveys are completed on the incidence of positive and negative reactions in various age groups and in groups of persons giving positive and negative histories of the disease, the complement-fixation test may be found to be of value in the detection of the susceptible individual.

It is evident, however, that the extensive application of the test will depend upon the discovery of a more readily available and cheaper source of antigen.

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A Method for Demonstrating an Antidiuretic Action of Minute Amounts of Pitressin: Statistical Analysis of Results.

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Using single rats rendered diuretic by the administration of water and alcohol, it has been possible to detect the antidiuretic action of as little as 0.02 milliunit or 20 microunits of pitressin given intravenously. The method is conservative of time and material. As an assay technic, it is at least as accurate as others heretofore reported.

Healthy male rats, of unselected stock, weighing 180-220 g were used. They were fed a diet of dog chow cubes, with biweekly additions of fresh vegetables. Prior to the test, they were starved for from 12-18 hours, but water was allowed *ad libitum*. At the time collections of urine were begun, the environmental temperature was brought to 29-30 C, and was maintained constant by the use of an electric reflector heater. Solutions to be administered by gavage were warmed to 40°C.

All containers used for handling pitressin had been carefully washed in sodium hypochlorite solution 5% (commercial), rinsed with hot and cold tap water and distilled water, and dried with alcohol and ether. Ampoules from a single lot of pitressin (No. 161) were

used. A convenient dilution was 1 part pitressin in 200,000 parts of physiological salt solution, where 1 cc = 0.1 μU or 100 microunits. Dilutions were made fresh for each day's experiment and were kept in the refrigerator. Samples removed for injection were used immediately. All injections were flushed through with 0.1 cc of physiological salt solution. The volume of fluid injected did not exceed 0.5 cc per dose.

An initial volume of alcohol-water solution was given by gavage, corresponding to 5% of the rat's weight. This serves as sufficient sedative. The alcohol water solution contained 12% of 95% alcohol. Thirty minutes later, 3% of the rat's weight of warm tap water was given by gavage. The animal was then fixed securely in the supine position. An intravenous needle (23 gauge, 1½ inches long) was introduced into the femoral vein, after incising the skin, and was clamped in place with its stilet inserted. The glass-tipped connection of a rubber cystostomy tube of 1/16 inch bore was tied into the urinary bladder through a suprapubic wound, after which the bladder was gently replaced and the wound was closed by a single suture. A ligature was tied to occlude the urethra. The collecting cylinder, graduated to 0.1 cc, was set on a stand allowing vertical movement, so that the outlet of the cystostomy tube remained approximately 7 cm below the animal's bladder throughout the experiment.

Measurements of cumulative urine volumes were made at about 10-minute intervals and were plotted on a graph, until three readings indicated that a steady rate of flow, in the range of 3.1-8.1 cc per hour, had been attained. This was usually from 90 to 120 minutes after the first gavage. The first injection of pitressin was then given. Readings were made at 4-minute intervals during the period of antidiuresis.

By subtracting the volume of urine secreted in the 20 minutes subsequent to injection, from that for the same period just prior to injection, a measure of the antidiuretic response (R) was obtained. A maximum of 4 injections was considered to be a feasible limit for a single animal. The interval between injections was 15-50 minutes.

Results. In Table I the effects of 53 injections of pitressin, utilizing 25 animals, are summarized. The series of means of (R) for each (log D) in the 53 observations are given in Table I and plotted as the dots in Fig. 1. It is evident that the relation between mean response and log dose is essentially linear over the range investigated.

TABLE I.
Dose Response Averages in 53 Injections.

Dose micro-units	20	40	80	100	160	200	320
Log Dose	1.30	1.60	1.90	2.00	2.20	2.30	2.51
Mean of R—cc	0.20	0.52	0.93	0.85	1.28	1.25	1.44
Variance of Mean	.0060	.0060	.0112	.0196	.0087	.0393	.0064
Number of observations	13	13	7	4	9	2	5

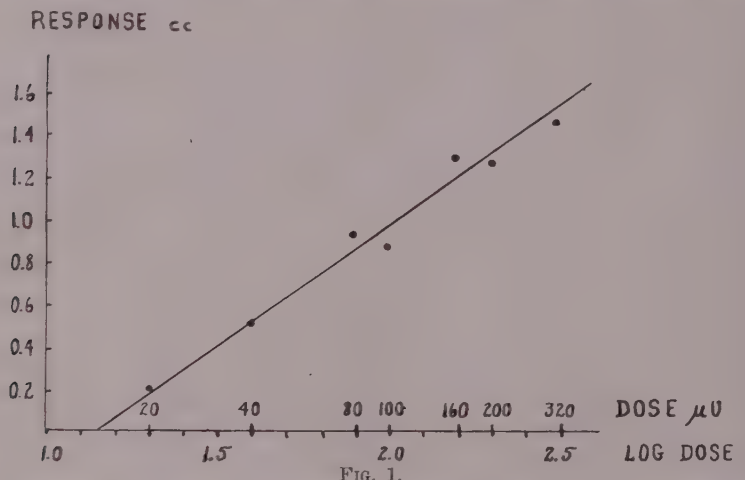

FIG. 1.

Pitressin Antiduresis. Illustrating the observed linear relation between log dose and antidiuretic response.

In the individual injections the variation of response-dose relation is high, with a standard deviation of (log D) from the expectancy of .28 (variance .08), indicating that 95% of the individual injections tend to give (log D) within ±.56 of the value predicted by the regression line. For Burn's method,[1] using his published data, the corresponding variance is .14 as compared with our .08; this difference is not statistically significant.

The regression of (log D) against (R) was calculated, weighting each mean by the reciprocal of its variance according to the usual statistical method. The resulting equation, for our data, is:

Expected (log D) = 1.137 + .90 R. This equation is shown graphically as the diagonal line in Fig. 1, the dots being the means of (R) for each dose.

Method of Assay. For assaying pitressin, or for comparing other antidiuretic substances with pitressin, it is necessary to evaluate both the potency of the particular lot of pitressin used as compared with that of our lot, and the variability in the animals' responses to iden-

[1] Burn, J. H., *Quart. J. Pharmacy and Pharmacol.*, 1931, **4**, 517.

tical doses of the new pitressin. This can be accomplished without the necessity for performing preliminary tests using pitressin alone, provided all injections of pitressin are given prior to administering other antidiuretic substances which might conceivably alter the effect of pitressin.[2] Our results indicate that it makes no important difference whether all injections of each preparation and of standard are given to one animal, or whether each preparation and standard is given to each of several animals. The order in which various doses are given should be governed by a method of deliberate randomization. The mean log-response relation must be determined for each lot of pitressin with at least as many injections as are used for the unknown. The slopes of our regression lines were not significantly different for the different rats with which we have worked, indicating the propriety of employing a regression line, or equation derived from averaging the data from all observations with standard pitressin.

Thus in assaying the difference between two preparations, an approximately equal number of doses of each preparation should be given to each of several animals, and in such relative doses that the mean response to each preparation will be approximately the same. Correction of the mean dose-response of one preparation to the mean response level of the other preparation can be performed by a shift parallel to one's average regression line, as illustrated in Fig. 1, or by the use of the corresponding coefficient, which is .90 for our data. The difference in log D for the two preparations at equal responses permits an evaluation of the ratio of potency.

The variance of the difference of (log D), for the two preparations at the same mean response, is the sum of 3 components: the variance of estimate of the mean dose at mean response for each of the 2 preparations, and the variance of the correction to equal response level. Its equation, using our observed variance (.08) for individual injections becomes:

$$\sigma^2 = \frac{.08}{n_1} + \frac{.08}{n_2} + .011\ (R_1 - R_2)^2$$

where n = the number of injections of a given preparation, and .011 is the variance of the regression coefficient of the slope (.90). The last term in the equation approaches 0, if the relative doses are selected so as to give equal mean responses for the two preparations.

The following example illustrates the calculation:

Prep. 1	Prep. 2
$n_1 = 3$	$n_2 = 3$
Mean $R_1 = 1.433$	Mean $R_2 = 0.767$

[2] Silvette, H., *Am. J. Physiol.*, 1941, **131**, 601.

Mean log $D_1 = 2.000$ Mean log $D_2 = 2.204$

Cor. mean log $(D_2) = 2.204 + .90\ (1.433 - .767) = 2.803$

Diff. log $D = 2.803 - 2.000 = 0.803$

$$\text{Var. of estimate of log } D_1 = \frac{.08}{3} = .027$$

$$\text{Var. of estimate of log } D_2 = \frac{.08}{3} = .027$$

Var. of cor. of mean log D_2 to response level $R_1 = .511\ (1.433 - .767)^2 = .005$

Var. of calculated diff. of log (D) at the same R $= .059$

Standard error $= .243$

This assay may be taken to indicate that the potency ratio of the two preparations lies between antilog $.80 \pm 2\ (.24)$ that is, Prep. 1 is between 2 and 20 times as potent as Prep. 2. If similar results had been obtained, using 20, instead of 3 injections, the ratio of potency would have been between 5-8, probably a usefully narrow range for assay work.

Summary. Single rats rendered diuretic by water, and alcohol in sedative dosage, constitute a satisfactory biologic test for the antidiuretic action of pitressin. It is 10 times more sensitive than Walker's[3] test, in which he used the diuretic rabbit; however, the required dose of pitressin per kilogram of animal is essentially the same. As an assay technic, using the same number of injections, ours is at least as reliable as the multiple rat test of Burn,[1] and as other tests available for antidiuretic substances.

[3] Walker, A. M., *Am. J. Physiol.*, 1939, **127**, 519.

All preliminary manuscripts are indicated by the letter P after the number of the article

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